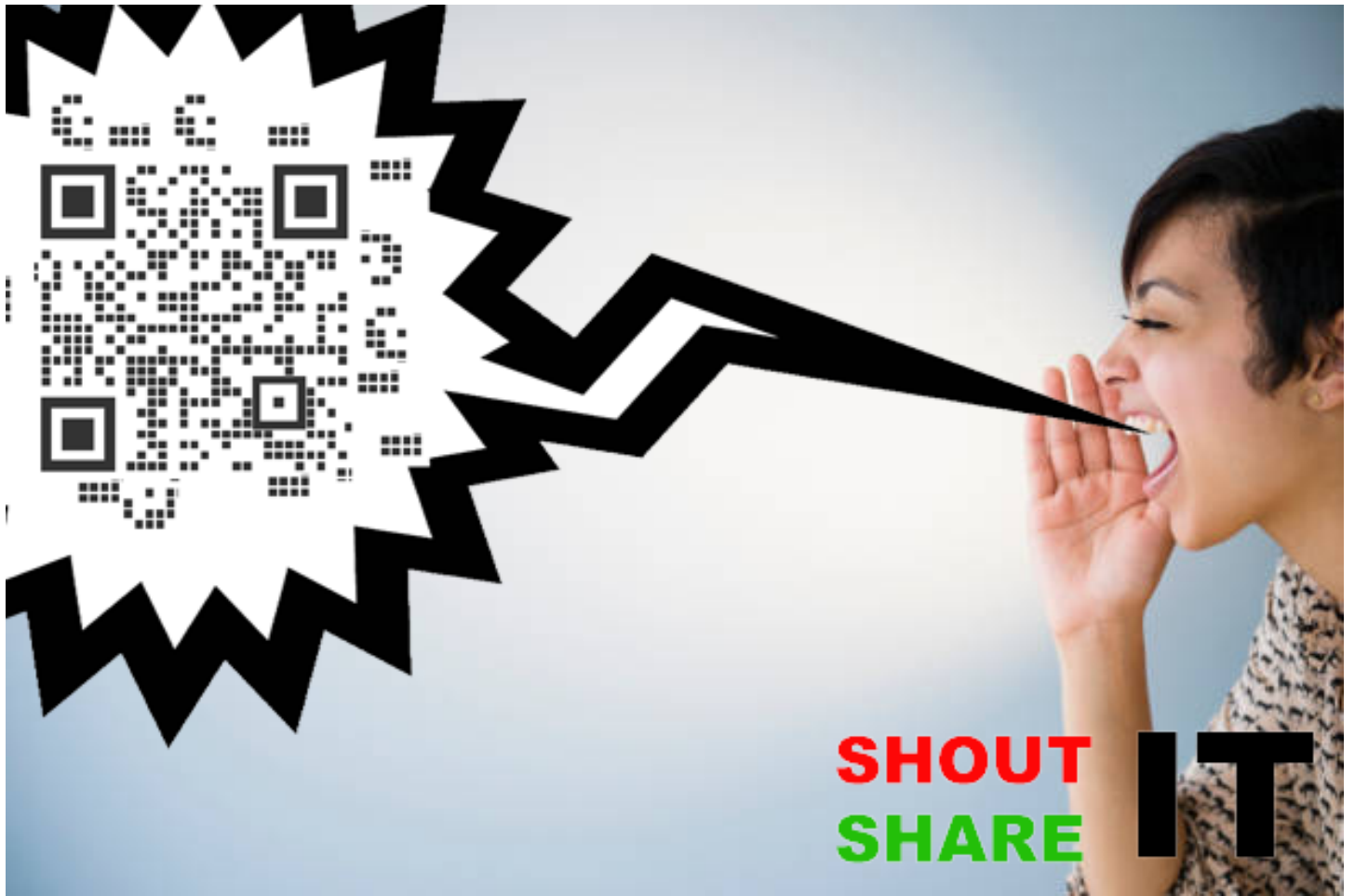




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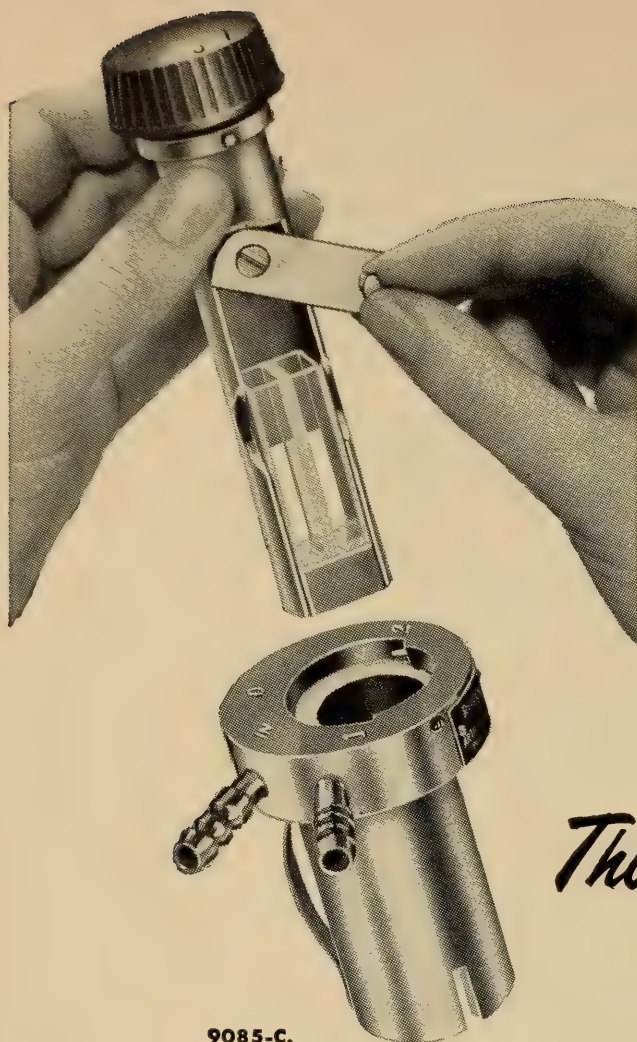
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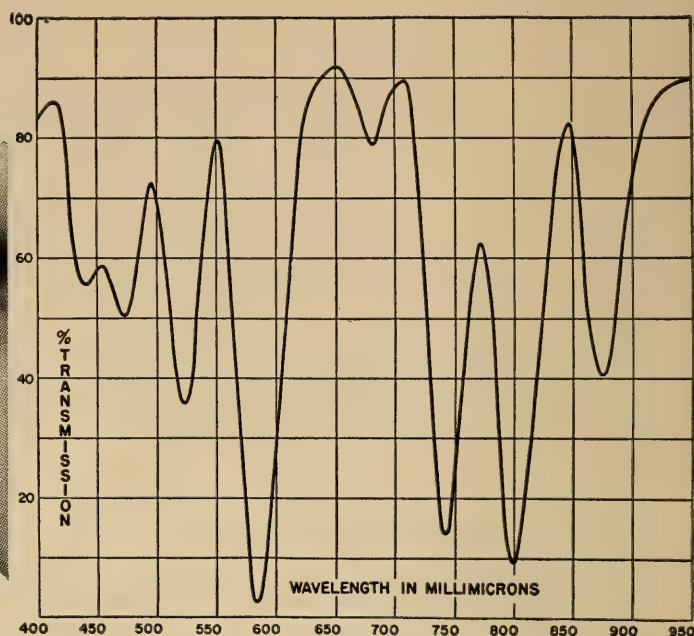
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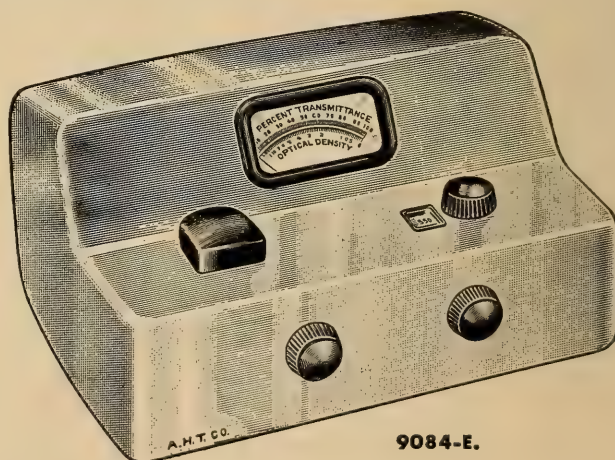
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THE BIOLOGICAL BULLETIN

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TAIL MELANOPHORES OF *XENOPUS* IN NORMAL DEVELOPMENT AND REGENERATION ¹

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Department of Zoology, University of Arizona, Tucson, Arizona

A peculiar group of light-sensitive melanophores occurs in the ventral fin of *Xenopus* larvae. Bles (1905) noticed that these chromatophores are contracted during the day, but expand at night, darkening the tail markedly. More recently, Bagnara (1957) has shown that the melanophores of the tail fin are directly photosensitive and refers to this response as the "tail darkening reaction." His suggestion that a photochemical system is involved has been supported by Van der Lek, De Heer, Burgers and von Oordt (1958) who point out that the degree of melanophore response is a function of light intensity.

Because of the above observations and in view of the report that the numerical density of tail fin melanophores is only slightly influenced by the hypophysis (Bagnara, 1957), it seems likely that the presence or absence of light, not the pituitary, is the primary effector of tail melanophore response. The chromatophrophic hormone (CTH) of the hypophysis exerts some influence on these cells, however, as was shown by Thing (1952) who used the tail fin melanophores for assay of such hormone preparations.

It is curious that these melanophores are restricted to the distal half of the ventral fin (Bles, 1905; Bagnara, 1957). Apparently, the tip of the tail provides an environment favorable not only for the establishment of a light-sensitive mechanism, but also for melanogenesis. That the caudal portion of the ventral fin is generally chromogenic is suggested by our previous observation (Bagnara, 1957) that guanophores, which are never present in the tail of normal *Xenopus* larvae, develop in the distal portion of such tadpoles which have been deprived of their hypophyses.

The present study concerns the responses of tail fin melanophores of *Xenopus* to changes in illumination during the course of normal development and regeneration, and compares these melanophores with those in other areas of the larvae.

MATERIALS AND METHODS

The larvae of *Xenopus laevis* used in this investigation were reared from eggs obtained from natural spawning of our adult stock which are kept in outdoor tanks.

¹ Aided by grants G-4048 and G-6231 from the National Science Foundation.

In a few cases, however, ovulation was induced with chorionic gonadotrophic hormone. Throughout the larval period, powdered nettle was employed for feeding. Experiments were carried out in a temperature-controlled room at temperatures of 21° C. during the spring and summer and 24° C. during the fall and winter. Illumination was from north-facing windows.

During some of the experiments, larvae were subjected to darkness for periods of thirty minutes to several hours. Thirty minutes is approximately the minimal time interval for achievement of the tail darkening reaction (Bagnara, 1957; Van der Lek *et al.*, 1958). Dark treatment was carried out in a closed drawer in a darkened room. After appropriate intervals of exposure to darkness, the larvae were immersed immediately in 25% formalin. Such treatment insures rapid fixation and prevents melanophore contraction during the fixing process. The same method of fixation was employed in all experiments in which degree of expansion was to be observed. For long-term preservation, the larvae were removed to 10% formalin.

For the tail regeneration experiments, young *Xenopus* larvae of stages 51 through 54 (stages of Nieuwkoop and Faber, 1956) were used because older animals usually conclude their metamorphosis before tail regeneration is completed. Tails were cut off with iridectomy scissors while the larvae were hovering in a stationary position. The cuts were made just anterior to the pigmented area. In this way, enough tail remained to allow the larvae sufficient mobility for feeding and for respiration. Care was taken to use only tadpoles on which none of the pigmented area of the fin remained.

In order to evaluate the degree of melanophore response, the melanophore index (M. I.) of Hogben and Slome (1931) was employed. This index is general enough to permit analysis even of types of melanophores which differ slightly from one another in form.

RESULTS

I. Normal development of the fin melanophores

Tail fin melanophores begin to appear at stage 47 (Fig. 1). They are first seen immediately adjacent to the somitic area at the very tip of the tail. The pigmented region gradually increases in size so that at stage 51 the distal third of the tail fin contains many melanophores. By stage 52 the pigmented area has increased to cover the entire caudal half of the tail. The anterior boundary of the pigmented region forms a clear line of separation from the proximal portion of the ventral fin which contains no melanophores at all.

In their early developmental stages, the young tail melanophores are elongated and sparsely branched. As differentiation proceeds, new branches are added radially so that the expanded melanophores appear stellate. During stages of expansion, melanophores of the tail fin differ from those of the head and trunk in that their processes are thin and much more heavily branched. As tail pigmentation proceeds during the early larval period, new melanophores are added at the edge and tip of the fin. Actually, there is a gradient in degree of differentiation; thus, under expanded conditions, melanophores immediately adjacent to the somites are fully developed and stellate while those at the edge are elongated (Fig. 2). Between these two areas exist partially differentiated melanophores

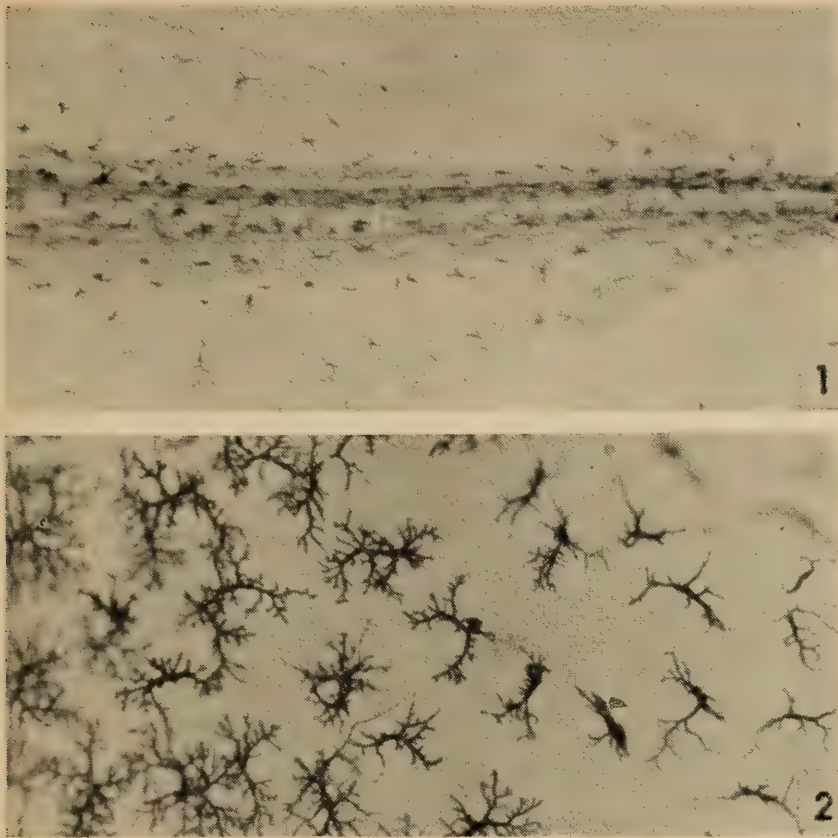


FIGURE 1. First appearance of tail fin melanophores at stage 47. Normal aquarium illumination. Magnification: $\times 10$.

FIGURE 2. Ventral fin of stage 53 larva during the tail darkening reaction. Melanophore differentiation gradient from somites at left to fin edge at right can be seen in these expanded melanophores. Magnification: $\times 50$.

with an intermediate number of branches. This suggests either that new melanophores are proliferating at the edge of the fin or that here unpigmented melanocytes are coming under the influence of a melanogenic substance which is distributed along a gradient from the somites to the ventral fin edge.

II. Normal development of the tail darkening reaction

Although not as striking as that of fully differentiated melanophores, the response of differentiating melanophores is discernible almost from their first appearance. Under normal room illumination, young melanophores are not able to assume the punctate form of the fully developed tail melanophores (Fig. 3); instead, they appear elongate with relatively few visible branches (Figs. 1 and 2). After exposure to darkness for thirty minutes or more, however, their branches become more obvious and often secondary branches can be seen (Fig. 2). Fully formed tail melanophores seen during the tail darkening reaction are stellate and possess secondary and tertiary branches (Fig. 4).

III. Regeneration of tail melanophores and the tail darkening reaction

Approximately four days after tail amputation, a new fin can be seen forming at the end of the regeneration blastema and at the cut edge of the ventral and

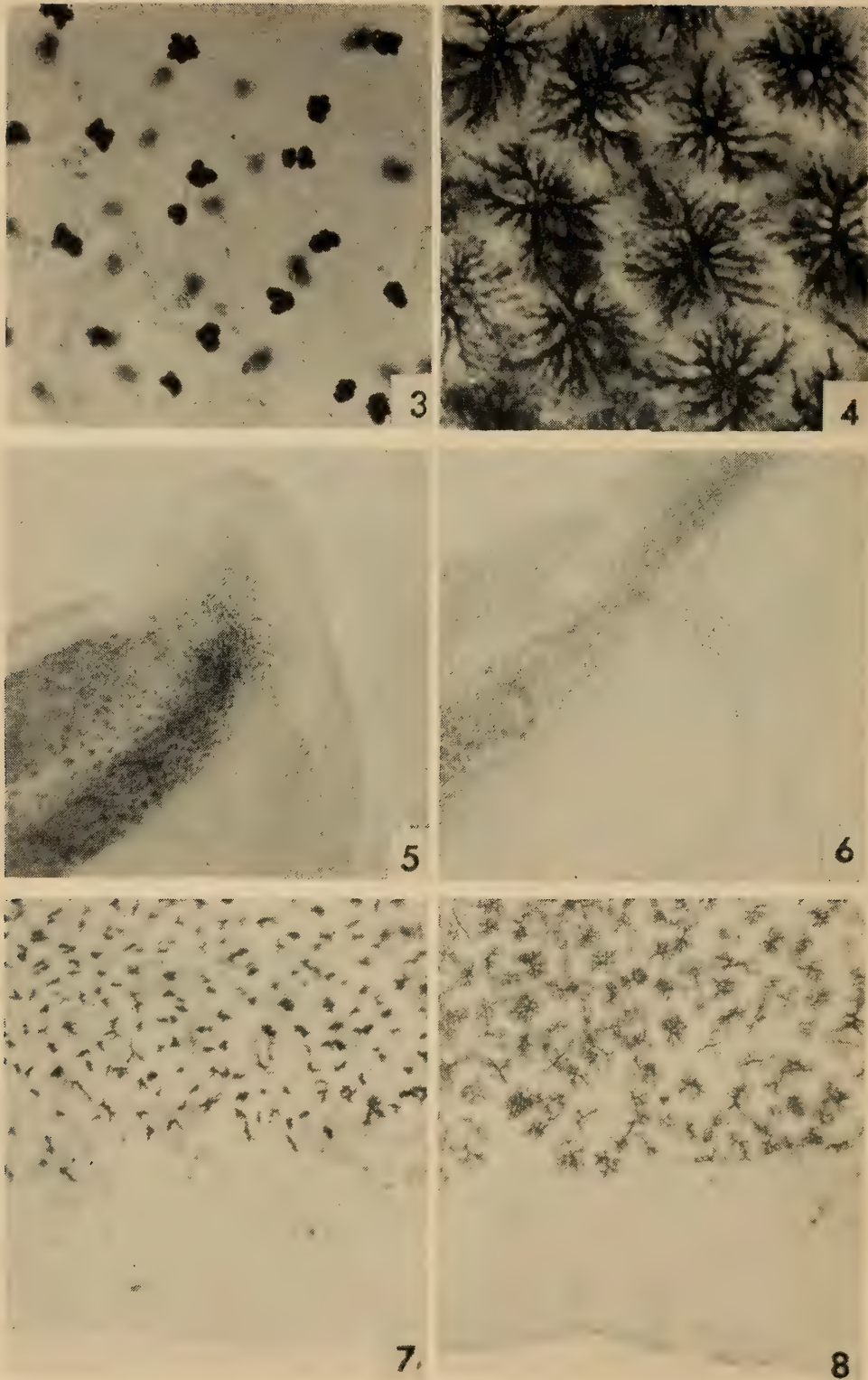


FIGURE 3. Mature melanophores of ventral fin in full contraction after bright illumination. Magnification: $\times 100$.

FIGURE 4. Mature melanophores of ventral fin in full expansion during the tail darkening reaction. Magnification: $\times 100$.

FIGURE 5. Fifth day of tail regeneration. Note melanophores streaming from the blastema at the line of amputation. Magnification: $\times 7$.

FIGURE 6. Seventh day of tail regeneration. Melanophore streaming much more obvious. Magnification: $\times 7$.

dorsal fins. At this time, the formation of melanophores begins in the new ventral fin near the base of the somitic blastema. During the next 6 or 7 days, these melanophores increase in number and appear as a stream of cells stemming from the regeneration blastema (Figs. 5 and 6). These melanophores are present only in the ventral fin and remain excluded from the area anterior to the original cut. Thus, the proximal area retains its unpigmented state. During the course of tail regeneration, tail melanophores gradually appear in the ventral fin, first making their appearance adjacent to the newly forming somites. After the tenth day of regeneration, the somitic core of the regenerating tail appears as a long spike to which is attached a generous ventral fin. Melanophores are abundant in the upper half of the fin near the developing somites, but are lacking in the lower half near the edge. After three weeks, tail regeneration is practically completed and the

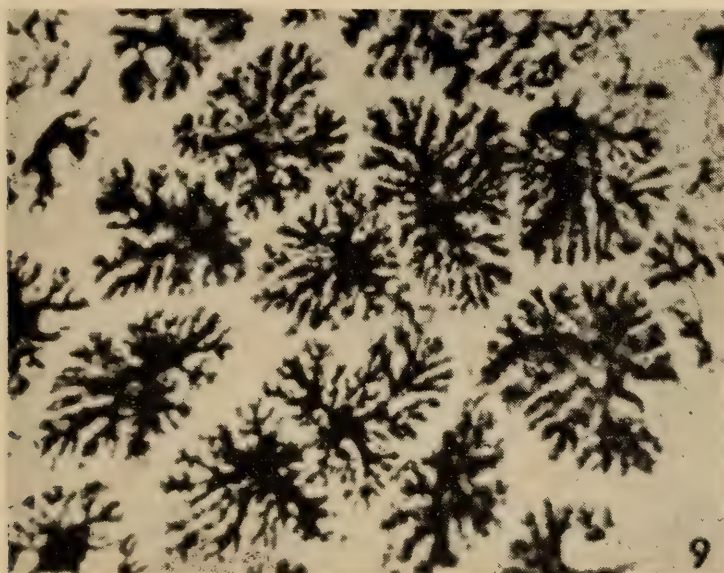


FIGURE 9. Melanophores typical of the head of mature larvae under normal illumination. Magnification: $\times 100$.

new ventral fin is copiously supplied with melanophores. However, a clearly defined margin is present along the ventral edge of the fin (Figs. 7 and 8) which is permanently devoid of melanophores. Moreover, the melanophores at the edge of this margin are completely differentiated and there is no indication of any newly forming melanophores which might ultimately invade the clear margin. The unpigmented border extends along the complete length of the regenerated ventral fin except for the extreme proximal portion of the pigmented zone. This is the point at which the stream of melanophores was first seen in connection with the regeneration blastema. Apparently at the start of regeneration, a sufficient stimulus for melanization allows the complete reconstitution of pigmentation in this localized region. Presumably, in later stages of regeneration, the melanizing

FIGURE 7. Unpigmented area at edge of ventral fin at twenty-first day of regeneration. Slight tail darkening reaction is evident because larva was preserved immediately after removal from a murky aquarium. Magnification: $\times 10$.

FIGURE 8. Larva similar to that in Figure 7. This larva, however, was placed in the dark before fixation; thus, the regenerated fin melanophores are fully expanded. Magnification: $\times 10$.

stimulus is not sufficiently strong for complete repigmentation and thus the clear marginal area is formed.

Just as in normal development, the fully differentiated melanophores in the regenerating fin are light-sensitive (Figs. 7 and 8). Under normal illumination, the melanophores are punctate in appearance; but after exposures to darkness of thirty minutes or more, they expand and assume a stellate form. Differentiating melanophores in the tail regenerate are also light-sensitive just as they are during normal development (Fig. 2).

IV. Observations on other melanophores

In contrast to the melanophores of the tail fin which expand upon exposure to darkness, the large epidermal melanophores of the head (Fig. 9) contract after similar treatment. This reaction was observed empirically by Bles (1905), but no quantitative estimate of the response was recorded. In the present experiments, it was observed that the average M. I. for head melanophores of 64 tadpoles which were kept under normal illumination was 4.7, while the M. I. for 74 tadpoles fixed after exposure to darkness was 3.3. This response to changes in illumination is not as marked as that shown by the melanophores of the tail fin but it is clearly visible even macroscopically on the tadpoles which, except for the tail, appear to blanch after exposure to darkness.

DISCUSSION

The fact that light-sensitive melanophores develop in the fin of *Xenopus* larvae poses several questions of embryological and physiological significance. First of all, what mechanism allows these peculiar light-sensitive melanophores to form only in the distal half of the fin, leaving the proximal portion completely free of this type of chromatophore? Stevens (1954) has shown that the posterior trunk tailfold areas of *Xenopus* neurulae are a good source of melanophores; thus the entire ventral fin has an adequate reservoir of melanoblasts upon which to draw. Realization of the melanophore potential is another matter, however, and may be dependent, as Twitty and Niu (1954) have suggested for urodeles, upon migratory responses of melanophores to environmental stimuli. It is possible, therefore, that the proximal area of the ventral fin does not provide an "attractive" stimulus for the migrating melanoblasts and that instead such cells migrate and complete their differentiation in the more "attractive" distal portion of the fin. Because of the peculiar melanophore distribution and because of the lack of other varieties of pigment cells in the tail, it is hard to explain the tail pigmentation patterns on the basis of physiological antagonisms between migrating chromatoblasts as has been suggested by Twitty and Niu (1954).

As an alternative explanation, it can be supposed that melanoblasts invade both the proximal and distal areas of the fin, but for reasons as yet unknown, only the distal portion of the tail provides a medium favorable for melanin synthesis. Wilde (1955) has strongly suggested that special metabolism of phenylalanine is required for normal differentiation of neural crest elements; thus, one might reason that prevalence of phenylalanine in the distal region may provide the stimulus for tail fin melanization. Furthermore, a gradient of this substance or some other melanogenic substance flowing outward from the somite to the edge

of the ventral fin would possibly account for the apparent differentiation gradient observed in the fin melanophores during normal development. Consistent with this hypothesis, the unpigmented margin along the edge of the regenerated fin may result from either a gradient which is too weak at its periphery, or from a diffusion blockage which does not allow penetration of the melanogenic substance to the edge of the fin.

Another fundamental question which suggests itself is concerned with the fact that not only are the tail melanophores extraordinarily light-sensitive but their responses are just the reverse of those of melanophores of other areas. The present observation that epidermal melanophores on the head and dorsal surface contract as a result of dark exposure is known for other amphibian larvae: for *Ambystoma*, Babak (1910) and Laurens (1917) and for *Taricha* and *Rana*, Bagnara (unpublished). To our knowledge, however, the very marked melanophore expansion, such as is seen in the tail of *Xenopus*, is unique among Amphibia. What mechanism allows the tail melanophores alone to possess this capacity is an enigma. Apparently the distal tail fin provides an environment which is favorable not only for melanogenesis but also for the development of a light-sensitive mechanism.

Whether the dorsal melanophores of *Xenopus* larvae are directly stimulated by light has not been determined; however, one is led to think that this is so because of the observations by Laurens (1917), who showed that the dorsal melanophores of blinded *Ambystoma* larvae behave toward light like those of intact animals. His additional observation that melanophore expansion after removal to light occurs much faster than the original melanophore contraction brought about by dark-treatment suggests that a photochemical reaction is involved in this system just as for the tail fin melanophores. Although light sensitivity of the dorsal melanophores is apparently a common thing among amphibian larvae, it should be emphasized that the reaction is relatively subtle, with changes of approximately 2 units of M. I. compared to a change of about 4 M. I. units for *Xenopus* tail melanophores.

Coincident with the unusual behavior of the tail melanophores of *Xenopus* is the unique form exhibited by these chromatophores. In an expanded state, their very thin branches lead one to suspect that they have less melanin than the thick melanophores on the dorsal surface. Perhaps this is a reflection of the apparent low sensitivity of the tail melanophores to the chromatotrophic hormone. Under normal lighting conditions, when the dorsal melanophores are expanded under influence of CTH, the contracted state of the tail melanophores implies that they are not sensitive to the circulating level of hormone. If the amount of hormone is raised by injection of CTH (Thing, 1952), the tail melanophores expand, strongly suggesting that the threshold level of response to CTH is higher for the tail melanophores than for those on the dorsal surface. That the tail darkening reaction is independent of hypophyseal stimulation was shown by Bagnara (1957) who pointed out that it occurs equally well in normal larvae, in hypophysioprivic larvae and in excised tails.

It is interesting to note that the response of the tail melanophores to light appears fairly early during the differentiation of these cells. Possibly it may even be present in melanoblasts before the synthesis of melanin. The reaction does not appear to be very strong in young melanophores; however, due to the relatively

small amount of melanin in these young cells, it could be fully developed and not appear so. It is significant that the melanophores of the regenerated tail can carry out the tail darkening reaction. This seems to indicate that the tail has regenerated completely, not only morphologically but physiologically as well.

SUMMARY

1. The peculiar light-sensitive melanophores in the ventral fin of *Xenopus* larvae first appear about stage 47. Gradually, pigmentation increases in the distal half of the fin, but the proximal portion remains free of melanophores throughout the larval period. New melanophores first become visible at the fin edge, with an apparent gradient of melanophore differentiation from somite to edge. The young melanophores are thin and elongated, but as differentiation proceeds they add new projections and become stellate. Even the youngest melanophores exhibit some degree of tail darkening in the absence of light, but the strongest response is displayed by the fully formed melanophores.

2. Four days after tail extirpation, melanophores are seen in the regenerating ventral fin. Pigmentation returns to all of the ventral fin except for a margin along the fin edge, with a "somite-to-edge" gradient again apparent. The new melanophores are of the typical tail fin type and are fully light-sensitive.

3. Melanophores on the dorsal surface differ markedly from those on the tail. The former are heavily pigmented and are apparently more sensitive to the chromatotropic hormone than the latter. Dorsal melanophores contract upon exposure to darkness and expand in the light. They are less reactive to changes in illumination than the tail fin melanophores.

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THE RELATIONSHIP OF REPRODUCTIVE TEMPERATURE AND THE GEOGRAPHICAL RANGE OF THE MARINE WOODBORER LIMNORIA TRIPUNCTATA¹

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The objective of the study was to determine the influence of temperature on populations of the wood-destroying isopod *Limnoria*. The genus, which is economically important, contains about 16 described species (Menzies, 1957; Menzies and Becker, 1957; Pillay, 1957). Geographically, the species of the northern hemisphere have been divided into boreal, *L. lignorum*; temperate, *L. quadripunctata*; temperate-tropical, *L. tripunctata*; and tropical, 5-7 species.

The species we have worked with, *Limnoria tripunctata*, is found around the world. It is a hardy species which survives and reproduces under laboratory conditions. Our interest has been population growth at temperatures which might be encountered by the species in nature. On the basis of our results, we believe that we can explain the known distribution of the species in terms of the effect of temperature on population growth.

The specimens studied were collected through the courtesy of Mr. Thomas P. May, manager at the International Nickel Company testing laboratory at Wrightsville Beach, North Carolina, where the species is particularly abundant. The animals were air-shipped to the laboratory in containers which were packed so that oxygen was not excluded.

PROCEDURE

The animals received at the laboratory were removed from the infected wood, and sorted for size and sex. For each culture, 25 males and 25 non-gravid females were placed in a dish containing sea water and a piece of presoaked pine. In all three experiments a total of 1050 animals was used. Non-gravid females were selected in order to keep each culture equivalent to the next. The use of females with eggs in different stages of development would have permitted errors in defining the reproductive temperature range and population growth at the various temperatures. In order to permit the animals to establish burrows in the wood, each culture was allowed to stand one week at room temperature (20-24° C.). Salinity was held within tolerable limits by addition of distilled water. Tolerable limits, 30-39‰, were determined by preliminary experimentation. Salinity data for the three experiments are presented in Table I.

Seven cultures were prepared for each experiment in the manner described and one of each was put into a controlled temperature box and kept for a period of 66 days. This time duration was selected in order to allow for the production of young, but not their maturation. The cultures were kept at approximately

¹ Contrib. No. 395.

0, 5, 10, 15, 20, 25 and 30° C. Usual temperature variation in the cultures was ± 1° C. Short-term larger variations did occur as a result of equipment and power failure. These occasional failures resulted in a gradual return to room temperatures. For over 99% of the time, temperature variation was within ± 1° C. The temperature means from the twice daily record are shown in Table I.

TABLE I
Salinity of culture water at the start and finish of each experiment

Original salinity	Experiment I June, July, Aug. 35.7‰	Experiment II Nov., Dec., Jan. 34.1‰	Experiment III May, June, July 35.0‰
Temperature		End salinities	
0°	37.0‰	34.2‰	34.5‰
5	36.8	34.1	33.7
10	37.2	34.0	33.6
15	37.9	33.6	35.1
20	38.1	33.8	33.8
25	37.6	33.8	33.2
30	38.4	32.5	34.6

Mean temperatures during each 66-day period

Experiment I June, July, Aug.	0.5° C.	5° C.	9.9° C.	15.1° C.	20° C.	25° C.	29.8° C.
Experiment II Nov., Dec., Jan.	0° C.	5° C.	10° C.	15.1° C.	20° C.	25.1° C.	29.9° C.
Experiment III May, June, July	1° C.	6° C.	11° C.	16° C.	21° C.	24° C.	29.5° C.

The measurements taken of the young at the end of the 66-day period are recorded in size class, based on pleotelson width. The size classes used were taken from Johnson and Menzies (1956).

RESULTS

The results of three experiments, each of 66 days duration, are presented in tabular form (Tables II and III). These are the number of young present at the end of each experiment, the largest size attained by the young, adult mortality and gravidity of surviving females. From these data the population change in 66 days has been determined (Fig. 1).

Cultures kept at 0° C. showed no reproduction and the highest adult mortality (86–100%). At 0° C. the animals were immobile, did not feed and starvation is the probable cause of death.

The 5° C. cultures also showed no reproduction and a high adult mortality (50–92%). The animals were feebly motile and showed slight feeding, but here again starvation is the probable cause of death.

The 10° C. cultures showed no reproduction and a lower mortality (0–64%).

In two experiments gravid females were present; however, only eggs were found, and no larva or young were produced in the 66-day period. The animals were sluggish but did feed.

The 15° C. cultures showed a reproductive rate between 0 and 6 young produced per female. We are confident that a female produces more than one brood

TABLE II

Degrees Centigrade.....	0°	5°	10°	15°	20°	25°	30°
Adult mortality—Number of individuals in each culture of 50 animals that died in 66 days							
Experiment I June, July, Aug.	46	25	6	3	8	7	7
Experiment II Nov., Dec., Jan.	50	46	32	20	27	26	32
Experiment III May, June, July	43	28	0	4	2	0	32
Number of young present at the end of 66 days							
Experiment I June, July, Aug.	0	0	0	152	302	457	254
Experiment II Nov., Dec., Jan.	0	0	0	0	43	64	9
Experiment III May, June, July	0	0	0	20	45	73	0
Largest size class attained by young in 66 days*							
Experiment I June, July, Aug.	—	—	—	2	3	3	3
Experiment II Nov., Dec., Jan.	—	—	—	—	2	2	2
Experiment III May, June, July	—	—	—	2	3	3	—

* Pleotelson Width Size Classes after Johnson and Menzies (1956).

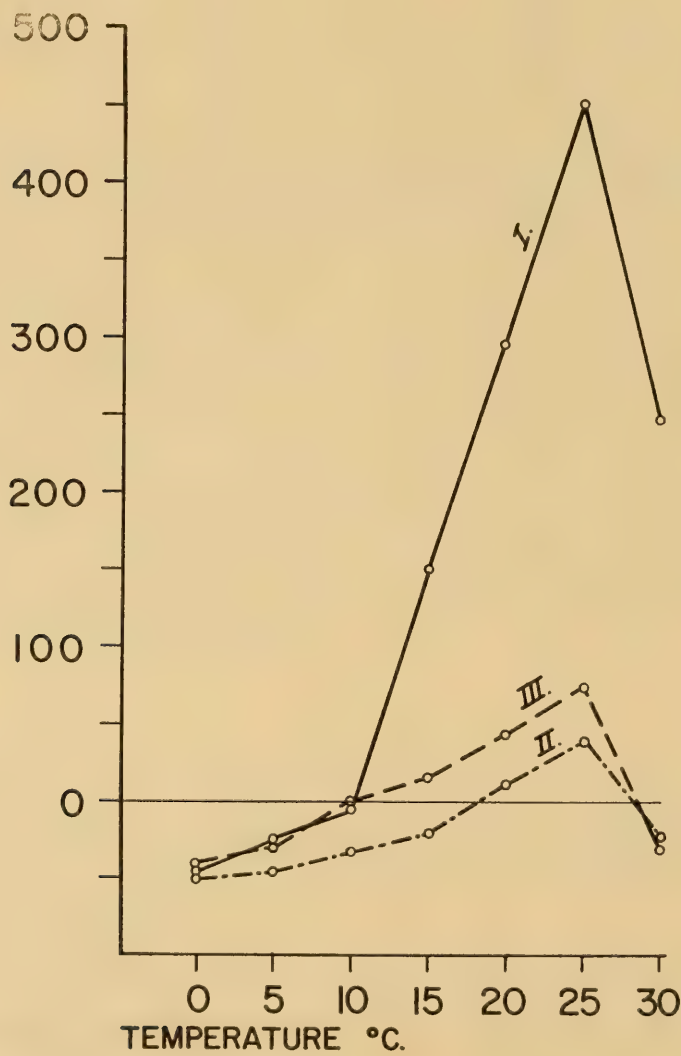
Class	Width in mm.
2	.28-.33
3	.35-.40

during her lifetime and therefore a 15° C. temperature permits population growth to occur. Adult mortality was 6-40%.

The 20° C. cultures showed a reproductive rate between 2 and 12 young per female. The average rate was 5 young per female and growing population is evident. Adult mortality varied between 4 and 54%.

TABLE III
Gravidity at the end of the experiments

Degrees C.	Experiment I June, July, Aug.			Experiment II Nov., Dec., Jan.			Experiment III May, June, July		
	No. females present	No. females gravid	Per cent gravid	No. females present	No. females gravid	Per cent gravid	No. females present	No. females gravid	Per cent gravid
0°	3	0	0	0	0	0	4	0	0
5°	9	3	33	1	0	0	12	0	0
10°	21	9	43	14	0	0	25	9	32
15°	16	9	56	16	0	0	22	1	5
20°	21	7	33	16	0	0	23	9	39
25°	22	7	32	18	1	6	25	9	32
30°	20	2	10	7	0	0	0	3	33



POPULATION CHANGE IN 66 DAYS

FIGURE 1. Population change at various temperatures after 66 days.

The 25° C. culture showed a reproductive rate between 3 and 18 young per female. The average rate was eight young per female, or about 1.6 times that of the 20° culture. The adult mortality rate was 0–52%.

The 30° C. culture showed a reproductive rate between 0.3 and 10 young per female. The average rate was three young per female. The adult mortality varied between 14 and 64%.

The most obvious feature of the three experiments is the lack of population growth below 10° C. The animals do not feed at these temperatures and eventually die of starvation as their fat reserve is depleted. The greatest population increase occurred in the cultures kept at 25° C. This might be considered the optimal temperature for population growth.

Adult mortality was highest at temperatures below 10° C. and at 30° C. Mawatari (1950) records 2–6° C. as the temperature lethal to adults of *Limnoria lignorum* (actually *L. tripunctata*). Our results also agree with the findings of Kampf (1957), who reports maximum population growth for *Limnoria tripunctata* from the Mediterranean to occur at 24° C. He also reports a shortening of the life span at higher temperatures which may account for the higher mortalities at 30° C.

INHERENT SEASONAL RHYTHM

Kampf (1957) reported a cyclic occurrence of gravidity for *Limnoria* kept under reasonably constant aquarium conditions. Unfortunately, his populations were mixed cultures of two species, *L. carinata* and *L. tripunctata*. Nevertheless, the low proportion of the former in his culture probably means that the cyclical record of gravidity is real for *L. tripunctata*. Coker (1923) reports that there is little winter gravidity found in Beaufort, North Carolina, *Limnoria* during the winter. Our observations of samples shipped from Wrightsville Beach, North Carolina, confirm this. The fact that culturing the specimens in the laboratory at temperatures approached only during the summer in the field still results in low gravidity and low young production suggests that we are dealing with an endogenous seasonal gravidity period which does not appear to be subject to immediate modification by change of temperature, and a reproductive rate imposed on the former which is subject to modification by change of temperature. It will be of the greatest interest to determine whether a seasonal endogenous reproductive period occurs in populations of the same species collected from a tropical locality where seasonal temperature fluctuations are almost absent.

DISTRIBUTION, TEMPERATURES AND POPULATION GROWTH

From the data presented, it would be a likely prediction that *Limnoria tripunctata* should not occur in localities where the sea water temperature is below 10° C. for most of the year. On the Atlantic coast the species is known to occur as far north as Cape Cod, Massachusetts. Here the yearly average sea water temperature is about 11° C. For five months of the year, however, the temperature rises above 15° C. and thus there is time for the populations to reproduce. The existence of the species is probably very precarious at this locality since monthly averages are below ten degrees for five months of the year. While signs

of *Limnoria* damage are extensive in the area, more often than not the burrows are empty and finding infested wood is sometimes a difficult job. North of Cape Cod, at Eastport, Maine, for example, the sea water temperature reaches 10°C . for only three months of the year. In such a situation we would not expect *Limnoria tripunctata* and to our knowledge the only species found there is *Limnoria lignorum*.

On the Pacific Coast of North America the species is not known as far north as Friday Harbor, Washington. Here the mean temperature of the surface sea water rises to $10\text{--}11^{\circ}\text{C}$. for only three months out of the year. During the re-

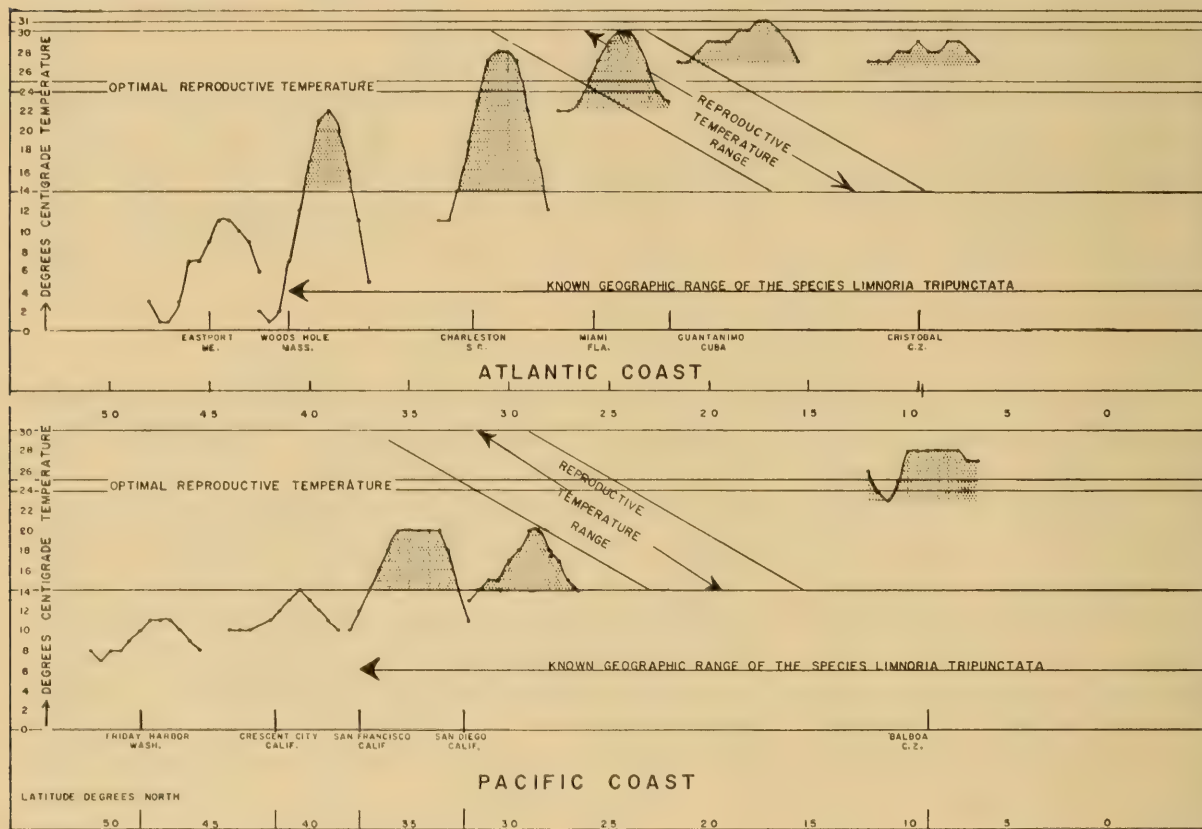


FIGURE 2. Geographical distribution, mean monthly surface sea water temperatures plotted at approximate longitude and reproductive temperature limits (the horizontal lines connected by the sloping block): The shaded areas represent the period of time that the temperature is within the reproductive range of the species. The minimum reproductive temperature, 14°C ., is estimated from the average of the three experiments to be at this temperature. At this point one young would be produced for each adult.

maining months it is lower than 10°C . This would give the species three months to feed, and no period of time suitable for reproduction. Southward, the species is also absent at Crescent City, California. Here temperatures rise above 10°C . for ten months of the year. The maximum temperature is 13°C . and this occurs for only two months of the year; 15°C . is not reached seasonally.

The species occurs along the Pacific Coast from San Francisco, California, to Mazatlan, Mexico, and probably as far south as Panama. At San Quentin, San Francisco Bay, lethal temperatures do not occur seasonally and temperatures favor-

ing population growth occur for seven consecutive months of the year. Seasonal temperature distribution, reproductive temperature limits for the species, and the established geographic distribution of *Limnoria tripunctata* in North America are shown in Figure 2. The remarkable coincidence between the geographic range of the species and the seasonal occurrence of temperatures favorable for reproduction leads us to conclude that temperature is a major factor controlling the distribution of the species.

The reason for the restriction of a species to a given geographic range has been of interest to zoologists and zoogeographers for many years. Temperature means, maxima and minima have been examined as parameters of possible control. The duration of a given range of temperatures has also been considered (Menzies and Hedgepeth, unpublished data). Experimentally determined physiological lethal limits for short periods of time are usually too wide to account precisely for the distribution of a marine species. Our examination of the effect of temperature on population growth of a single marine species suggests that the geographic distribution of this species is closely tied to the prevalence of temperatures favoring reproduction. The minimum duration of a favorable temperature necessary would depend on the growth rate of the species. Doubtless the duration of unfavorable temperatures for survival is also important. In terms of our experimental data it is difficult to explain the survival of populations of *Limnoria tripunctata* at Woods Hole, where it is necessary for the species to maintain itself for three months of the year at mean temperatures below 5° C. Our data suggest that this is a remote possibility; nevertheless, the species is precariously established.

The figures were drawn by Carolyn Peppin and Don Robinson. This research was sponsored by the U. S. Navy, Office of Naval Research, Contract Nonr-266 (41).

SUMMARY

1. Experimental results show that *Limnoria tripunctata* will feed at temperatures ranging from approximately 10° C. to 30° C. The reproductive temperature range appears to be from about 15° C. to 30° C. and the greatest population increase is in the neighborhood of 25° C. Excessive mortality results at 30° C.

2. Gravidity and hence number of young produced depends upon season and does not appear to be immediately modified by the presence of favorable reproductive temperatures.

3. The experimental results agree well with the known geographic range of the species.

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THE LARVAL STAGES OF PLEURONCODES PLANIPES STIMPSON (CRUSTACEA, DECAPODA, GALATHEIDAE)

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The zoeal larval stages of a galatheid crab were found in large numbers (up to 42,000/1000 m.³) in plankton tows taken at several stations of the Marine Life Research program of the California Cooperative Oceanic Fisheries Investigations near Magdalena Bay, Baja California, Mexico. Since these unknown larvae were found in an area where the galatheid *Pleuroncodes planipes* was extremely abundant, it seemed likely that they were the zoeal stages of that species.

METHODS

The larvae were tentatively identified as *P. planipes*. This identification was later confirmed by two methods: (1) the first larval stages were hatched from the eggs of the adult *P. planipes*, (2) one specimen of the fifth larval stage was caught in the process of molting to the juvenile stage, and this juvenile could be identified as *P. planipes*. Several attempts were made to follow the succession of larval molts in the laboratory. Hatching the larvae from the egg was relatively easy, and was achieved by several methods. However, no larvae survived longer than eight days, and all died before they had molted once. Plunger jars and still-water aquaria were used with variations of temperature, aeration and food (*Platymonas*, *Chlamydomonas* and *Coccolithophores*). The plunger jar method and a procedure described briefly by Ray (1958; p. 501), involving rotating flasks, apparently failed because of the constant vigorous tumbling of the larvae.

Adult females carrying eggs have been found in the plankton from December through March. Gravid females kept in the laboratory hatched larvae during most of that period and the same is probably true of females in nature. As larvae of all stages are found in the plankton from January to July, it has proved impossible to determine rates of development.

DESCRIPTION OF THE LARVAE

Stage I (Figure 35)

First antenna (Fig. 1). This appendage has a long, unsegmented base with two distal branches. These, the exopodite and endopodite, are continuous with the base. The former bears six setae, the latter, a long plumose spine.

Second antenna (Fig. 6). The antenna consists of a base, continuing into a long medial prickly spine, and a scale. The scale bears about eight plumose setae and is continued as an apical spine, prickly at the terminal end. On the

¹ Contribution from the Scripps Institution of Oceanography, New Series.

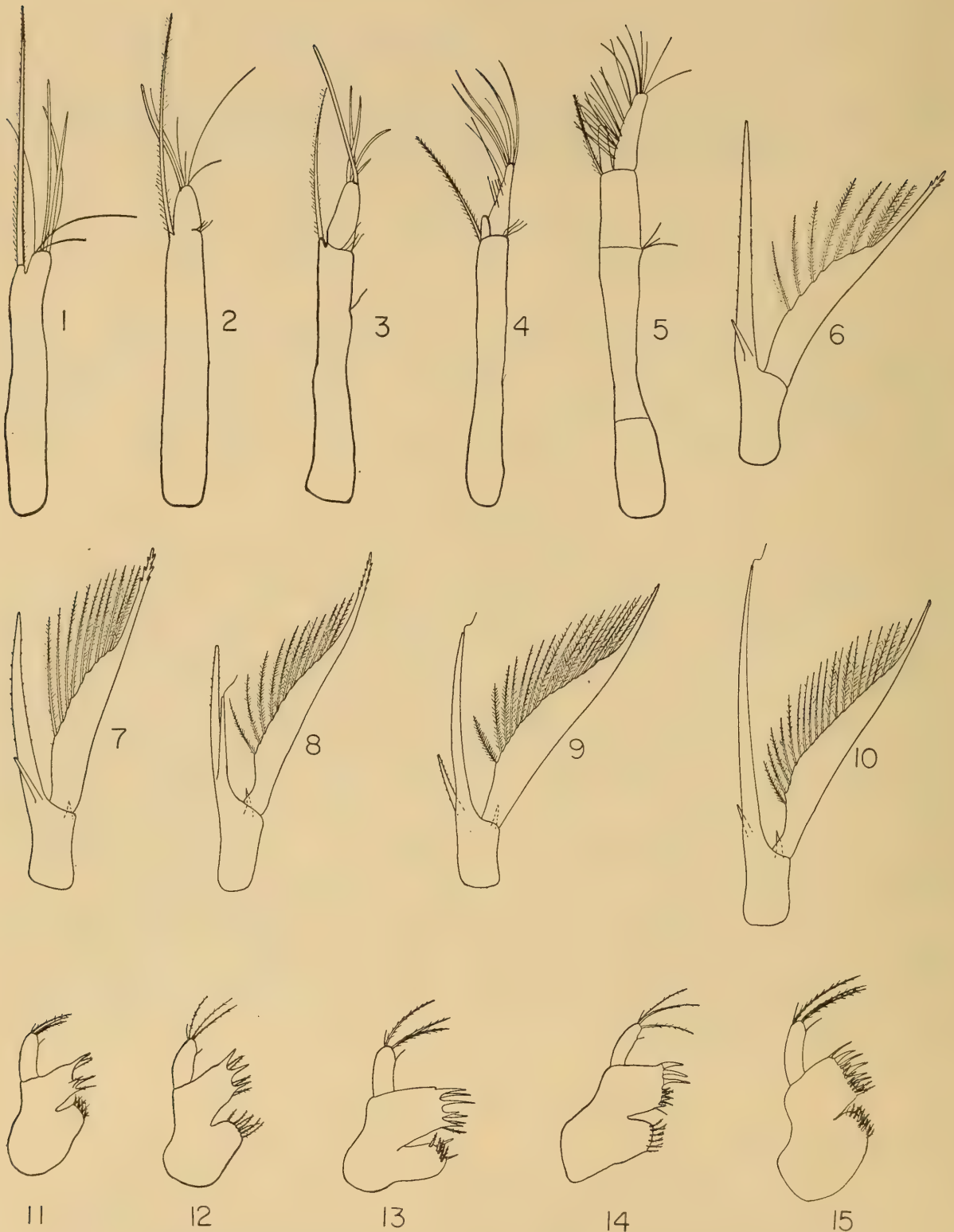
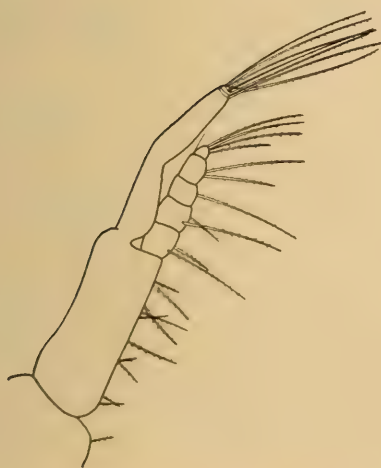
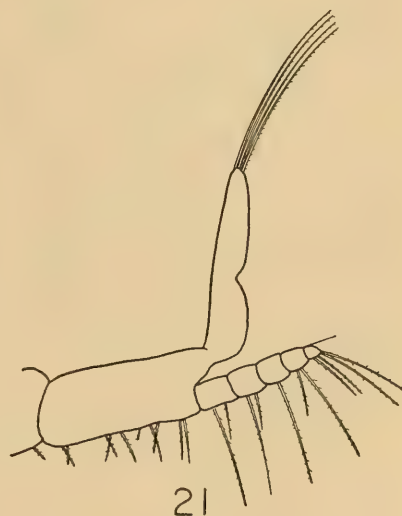
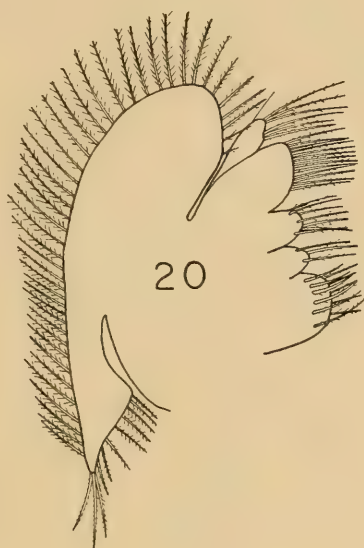
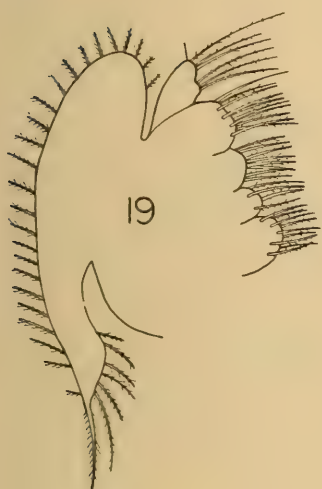
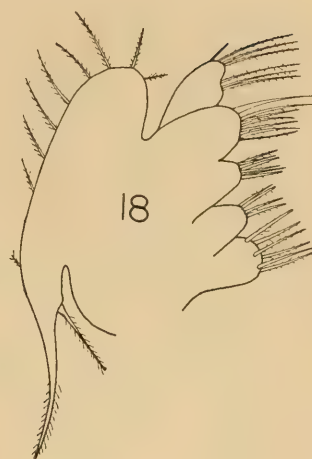
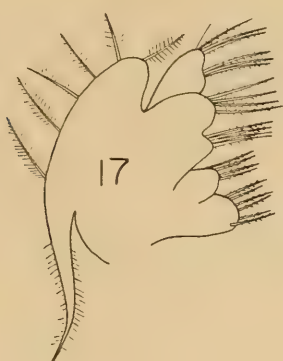
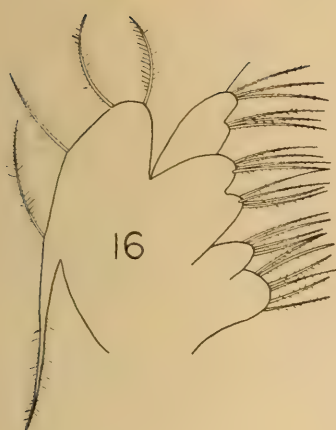


FIGURE 1. First antenna, Stage I.
 FIGURE 2. First antenna, Stage II.
 FIGURE 3. First antenna, Stage III.
 FIGURE 4. First antenna, Stage IV.
 FIGURE 5. First antenna, Stage V.
 FIGURE 6. Second antenna, Stage I.
 FIGURE 7. Second antenna, Stage II.
 FIGURE 8. Second antenna, Stage III.

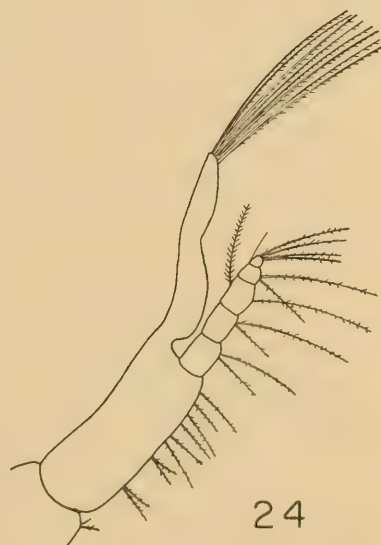
FIGURE 9. Second antenna, Stage IV.
 FIGURE 10. Second antenna, Stage V.
 FIGURE 11. First maxilla, Stage I.
 FIGURE 12. First maxilla, Stage II.
 FIGURE 13. First maxilla, Stage III.
 FIGURE 14. First maxilla, Stage IV.
 FIGURE 15. First maxilla, Stage V.



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FIGURE 16. Second maxilla, Stage I.
FIGURE 17. Second maxilla, Stage II.
FIGURE 18. Second maxilla, Stage III.
FIGURE 19. Second maxilla, Stage IV.
FIGURE 20. Second maxilla, Stage V.

FIGURE 21. First maxilliped, Stage I.
FIGURE 22. First maxilliped, Stage II.
FIGURE 23. First maxilliped, Stage III.
FIGURE 24. First maxilliped, Stage IV and V.

dorsal side of the inner flagellum is a smooth spine which, though small in this stage, increases in size in later stages.

Mandible. The mandible is a simple toothed process lacking a palp.

First maxilla (Fig. 11). The endopodite is a simple unjointed structure bearing three spinose setae and two smooth setae. The endopodite does not change in number of setae but only in size in the next four stages. The exopodite is split into a basipodite and a coxopodite. These generally add setae in the later stages. In Stage I, the basipodite bears five heavy setae. The coxopodite has seven setae.

Second maxilla (Fig. 16). As in the endopodite of the first maxilla, the endopodite of the second maxilla does not change the number of setae in any of the stages. Terminally on the endopodite there are four setae; three spinose and one naked. A group of three spinose setae is located subterminally. The basipodite lobes each bear four spinose setae, as does the distal lobe of the coxopodite. The proximal lobe of the latter carries six spinose setae. The scaphognathite carries only four plumose setae along its margin. Its posterior end is produced into a long plumose projection.

First maxilliped (Fig. 21). The maxilliped is well developed in the first larval stage. The appendage consists of a coxopodite with one seta on its interior margin, an unsegmented basipodite, an endopodite with five segments, and an exopodite with two indistinct segments. The basipodite has ten spinose setae on its interior margin. There are four setae terminally on the exopodite. The five segments of the endopodite each bear spinose setae, but in differing numbers. The fourth, second, and first segments have two setae, and the third but one seta. There is also one fine, smooth seta coming off from the joint of the distal (fifth) segment.

Second maxilliped (Fig. 25). The second maxilliped has much the same structure as the first. The endopodite of the second maxilliped, however, has only four segments; the third is elongated. The coxopodite has no setae; the basipodite has three spinose setae. The terminal (fourth) segment of the endopodite has four spinose setae and one fine, smooth seta. There are four setae terminally on the exopodite.

Third maxilliped (Fig. 29). The third maxilliped is rudimentary in Stage I; it lacks setae and is poorly developed in musculature. The appendage consists only of a basipodite and an exopodite; the endopodite is not present.

Abdominal segments. There are five segments, plus the telson in Stages I and II.

Telson (Fig. 36). The telson of the first larval stage has seven projections on each side. The first (outermost) is a spinose extension of the telson, and is unjointed at its base. This spine has 6-10 thorns distally. The second projection is a fine, hair-like, plumose seta. The next five are similar in that they are all plumose setae. In addition to the plumes, each seta carries thorns on its inner and outer margins.

Along the posterior margin of the telson between the plumose setae, several small thorns occur. In Stage I larvae there are usually one to three thorns between each of the inner five setae. Generally the number of thorns increases between the setae more medially located, so that there are usually two to three thorns between setae #6 and #7. In the medial cleft of the telson, one to three thorns

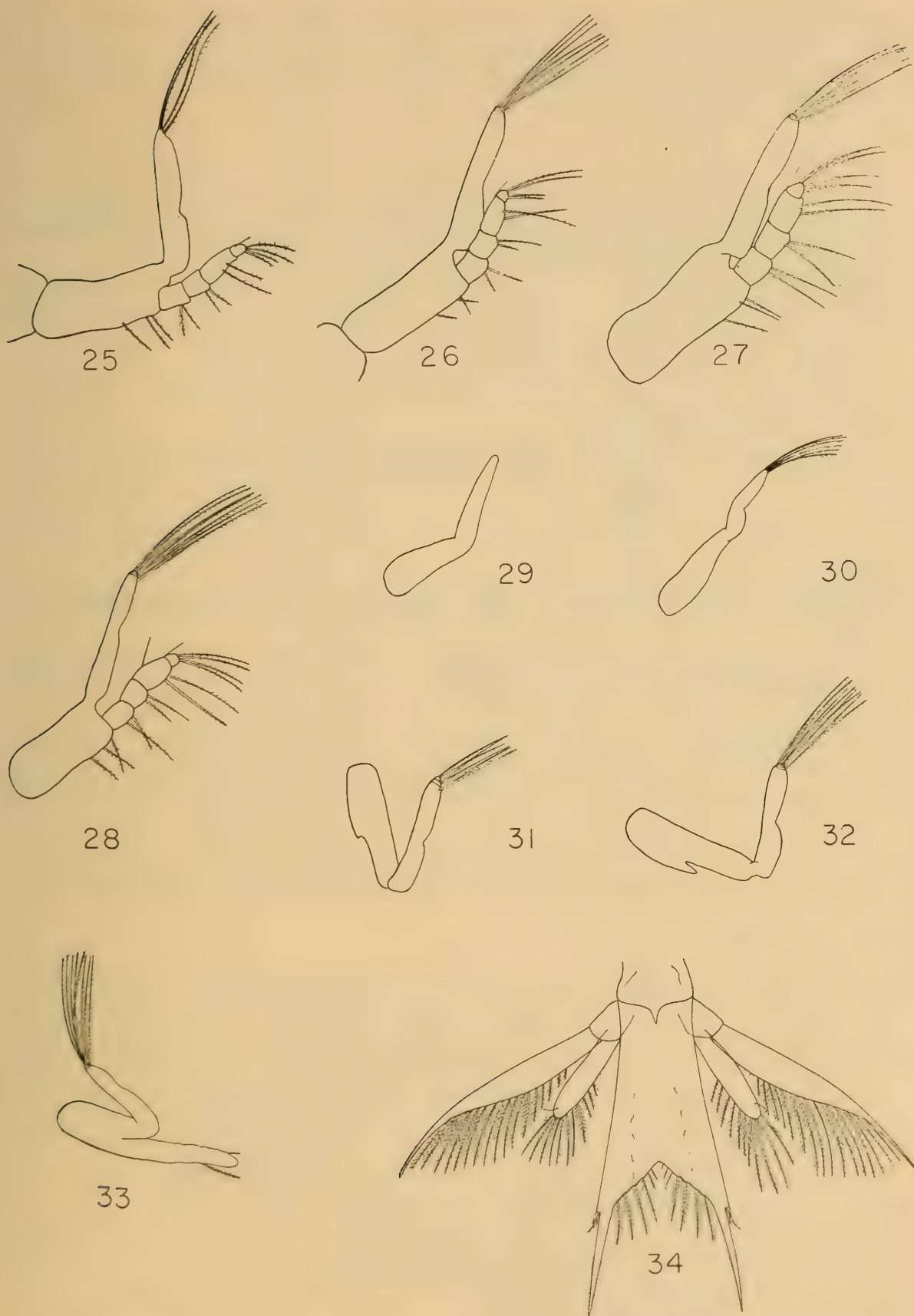


FIGURE 25. Second maxilliped, Stage I.
 FIGURE 26. Second maxilliped, Stage II.
 FIGURE 27. Second maxilliped, Stage III and IV.
 FIGURE 28. Second maxilliped, Stage V.
 FIGURE 29. Third maxilliped, Stage I.

FIGURE 30. Third maxilliped, Stage II.
 FIGURE 31. Third maxilliped, Stage III.
 FIGURE 32. Third maxilliped, Stage IV.
 FIGURE 33. Third maxilliped, Stage V.
 FIGURE 34. Telson, Stage IV.

may occur on each side. A tuft of short fine hairs occurs further medially. Each tuft is composed of seven to ten hairs. The numbers of these thorns and hairs are variable and are seldom identical between the right and left halves of the telson of any individual.

Stage II (Figure 37)

First antenna (Fig. 2). This appendage is much the same as in Stage I, but here three small hairs appear toward the distal end of the base. These hairs are on the location of an ultimate segmentation appearing fully in Stage III.

Second antenna (Fig. 7). The antennal scale has gained two more plumose setae, giving it usually ten. A new spine has appeared on the ventral side of the base. This spine is not segmented from the base, but is common with it.

Mandible. No change from Stage II.

First maxilla (Fig. 12). A seta has been added to the basipodite. The coxopodite has remained unchanged.

Second maxilla (Fig. 17). The endopodite, basipodite and coxopodite are unchanged. The scaphognathite has added two marginal setae, giving a total of six.

First maxilliped (Fig. 22). There are now seven terminal setae on the exopodite.

Second maxilliped (Fig. 26). There are now seven terminal setae on the exopodite.

Third maxilliped (Fig. 30). This appears to have become a functional organ; its musculature has developed and there are six setae on the terminal end of the exopodite.

Telson (Fig. 38). The primary change in the telson of Stage II larvae is the addition of one plumose seta, increasing the number of projections to eight. In this stage, however, the third projection does not have thorns on its margins, but only plumes.

The arrangement of thorns occurring between setae is similar to that of Stage I. In Stage II, however, there are neither hairs nor thorns in the medial cleft.

Stage III (Figure 39)

First antenna (Fig. 3). The segmentation initiated in Stage II has been completed, setting the exopodite off from the base. Three hairs originate at the joint. A hair appears on the exterior lateral margin of the base. The combined length of the new terminal segment and the setae is longer than the spine of the endopodite.

Second antenna (Fig. 8). The antennal scale now usually has 11 setae. The spine on the inner flagellum has increased in length and now has a terminal hair.

Mandible. No change.

First maxilla (Fig. 13). The basipodite has eight setae, an increase of two setae over Stage II. The coxopodite is unchanged.

Second maxilla (Fig. 18). The distal lobe of the basipodite now has seven setae. The proximal lobe has six. The coxopodite is unchanged. The exterior margin of the scaphognathite now has nine setae, plus one seta located on the interior margin of the posterior end.

First maxilliped (Fig. 23). There are now eight setae on the exopodite.

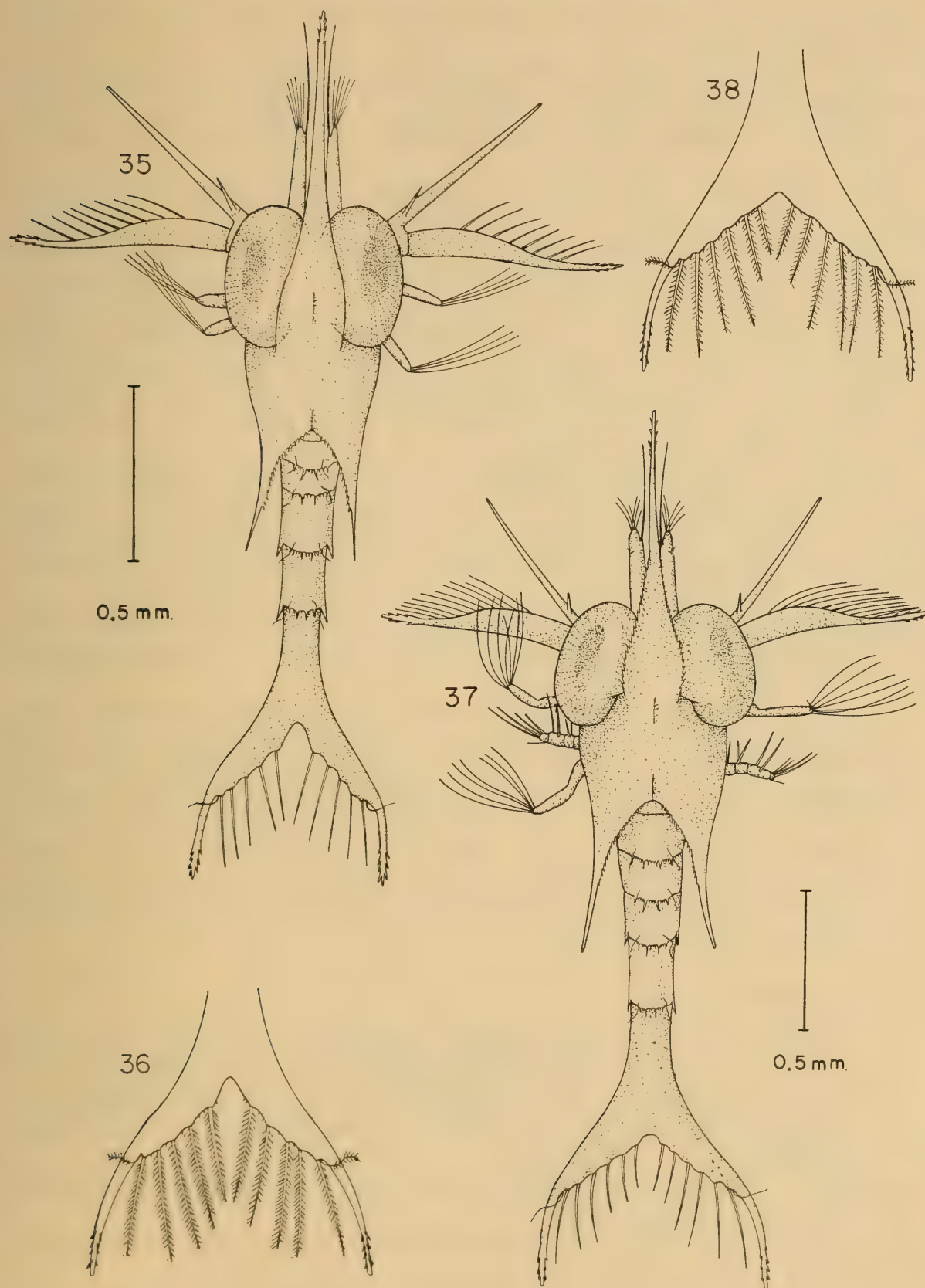


FIGURE 35. Larva, Stage I.
FIGURE 36. Telson, Stage I.

FIGURE 37. Larva, Stage II.
FIGURE 38. Telson, Stage II.

Second maxilliped (Fig. 27). The exopodite has added one terminal seta, giving a total of eight.

Third maxilliped (Fig. 31). The only change in the third maxilliped is the development of a small bud on the basipodite.

Abdominal segments. There are six segments, plus the telson in Stages III, IV and V.

Telson (Fig. 40). The changes in the telson between Stages II and III are radical. There are typically now nine projections on each half of the telson. The first (outermost) is a short naked spinose extension of the body of the telson. The second is a plumose hair-like seta. The third is a short heavy plumose seta. The fourth is the major projection of the telson; it is an extension of the telson and is not jointed at its base. It is covered on its interior and exterior margins by many small thorns. The fifth through the eighth projections are plumose setae bearing thorns on their lateral margins. The ninth projection is a small plumose seta lacking thorns. This innermost seta is not always present. It may be lacking on one or both sides of the telson.

Thorns are found abundantly between the fourth through the eighth projections. Between projections #4 and #5, zero to three thorns may occur. Usually three to five thorns are found between each of the fifth through eighth projections. Between the eighth projection and the medial line up to four hairs may be present, in addition to the ninth projection. The occurrence of these hairs is erratic.

On the dorsal surface of the telson, two pairs of very fine setae are found. The posterior pair is near the base of the seventh projection. The second pair is in line with the first pair, but anterior to it.

Uropods. The inner uropods are present only as poorly developed plates; they carry no setae. The outer uropods have from 8 to 11 plumose setae. The setae do not carry thorns. Their number is variable and may differ between the right and left sides. The tip of the outer uropod is not produced in an extended spine, but is short and naked. Small accessory setae commonly occur on the ventral surface of the outer uropod. These are not at the margin but are based somewhat anteriorly of the posterior margin. Generally there are two on each uropod, one between setae #1 and #2 (counting from the distal end) and the second between setae #4 and #5.

Stage IV (Figure 41)

First antenna (Fig. 4). The exopodite has gained three pairs of setae along its inner margin. In addition, the endopodite has developed a joint at its base.

Second antenna (Fig. 9). The scale usually now has 17 plumose setae. The spine on the inner flagellum has now increased in length so that it is much longer than the flagellum.

Mandible. The mandible at this stage has a small bud on the anterior margin which will become a palp in Stage V.

First maxilla (Fig. 14). The basipodite has 11 setae. The coxopodite has 10-11 spinose setae.

Second maxilla (Fig. 19). The lobes of the basipodite and coxopodite have added setae, but their absolute numbers are variable. The exterior margin of the scaphognathite now has about 25 setae; the posterior interior margin has six setae.

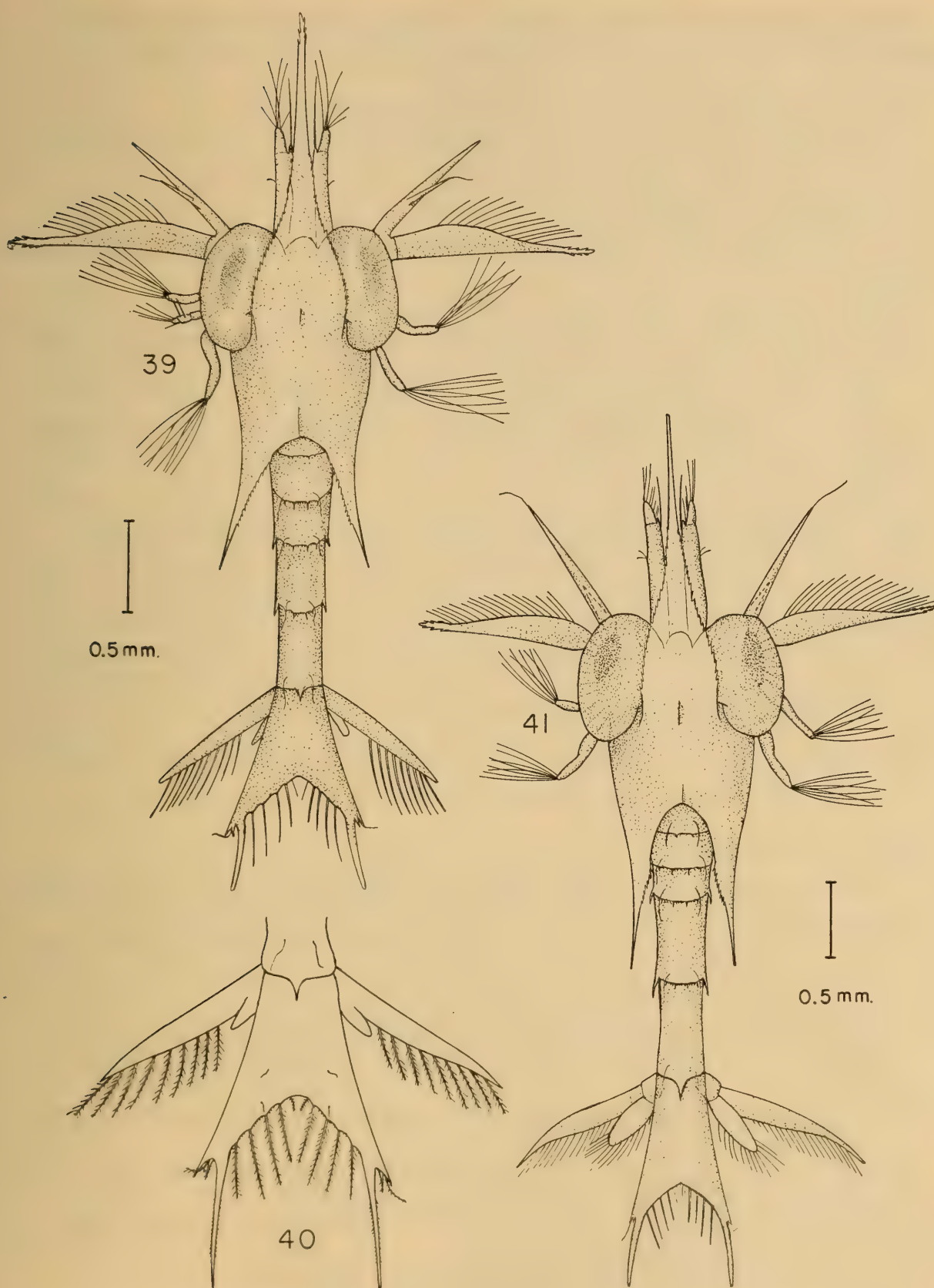


FIGURE 39. Larva, Stage III.
 FIGURE 40. Telson, Stage III.
 FIGURE 41. Larva, Stage IV.

First maxilliped (Fig. 24). The only change from Stage II and III is in the addition of a plumose seta on the exterior margin of the third segment of the endopodite.

Second maxilliped (Fig. 27). No change from Stage III.

Third maxilliped (Fig. 32). The exopodite has added two setae, giving now a total of eight. The bud on the basipodite has somewhat increased in size from Stage III, but is still rudimentary.

Thoracic legs. Thoracic legs are not visible on Stage IV larvae from a lateral view. They are present, however, as poorly developed appendages under the carapace.

Telson (Fig. 34). The telson in this stage is similar in aspect to that in Stage III, but it has added several plumose setae. The total number of projections may vary from 10 to 12, with the deletions occurring in the innermost setae; i.e., setae #11 and #12 may individually or both be absent. Typically there are 11 projections. Setae #9, #10, #11, and #12 are plumose but do not bear thorns. Projection #1 appears to be jointed and is no longer common with the body of the telson.

On the dorsal surface of the telson there are now five pairs of fine, hair-like setae. Three pairs have been added anteriorly to those present in Stage III.

Uropods. Inner uropod: this now has from 10 to 17 plumose setae. Three tufts of hairs occur medially from the innermost seta. A fine, hair-like accessory seta is found on the dorsal surface of the distal end of the inner uropod. Outer uropod: the extremity of this branch is now extended as a long spine bearing many thorns along its lateral margins. From 13 to 21 plumose setae are present on the posterior margin of this branch. Several fine hairs are found medially of the innermost seta. Four accessory setae are again found on the ventral surface of the outer uropod. These commonly occur between setae #1 and #2, #4 and #5, #9 and #10, and #14 and #15, starting from the outer seta.

Stage V (Figures 42-44)

First antenna (Fig. 5). The exopodite has added more medial setae, bringing the total to about 22. The endopodite has elongated, but is still shorter than the exopodite. The base now appears to be very lightly segmented into three parts.

Second antenna (Fig. 10). The number of plumose setae on the scale has increased to usually 19. The inner flagellum is reduced to a very small size and the spine which formerly grew upon it is as long as the antennal scale.

Mandible. The palp on the anterior margin is well developed, though unsegmented.

First maxilla (Fig. 15). The basipodite has about 15 setae and the coxopodite has about 13 setae.

Second maxilla (Fig. 20). The basipodite and coxopodite have added setae. The scaphognathite now has about 43 plumose setae on its exterior margin. Its posterior interior margin now has eight setae. The extreme posterior spine has been replaced by three plumose setae.

First maxilliped (Fig. 24). Unchanged from Stage IV.

Second maxilliped (Fig. 28). The only change from Stage III and IV is in the addition of a smooth seta on the exterior margin of the third segment of the endopodite.

Third maxilliped (Fig. 33). At this stage the endopodite appears. However, it is soft, poorly muscled and unsegmented. On its interior margin is a plumose seta and on its exterior margin, distally, is a fine smooth seta.

Thoracic legs. The thoracic legs are easily visible in a lateral view of the Stage V larvae. The first is chelate, the fifth is quite small and hidden behind the fourth leg.

Pleopods. There are now four pairs of unequally bifid pleopods present.

Telson (Fig. 43). The telson of this stage has reached its full development and variability of spine count is reduced. There are now commonly 12 projections. Of the inner eight projections, only #5 through #8 have thorns as well as plumes on their margins. One to five thorns are found on the margins of the telson between each of projections #4 through #9. No thorns are found medially of the ninth projection, but up to three hairs may occur between each of the setae between the ninth projection and the medial line. The five pairs of fine dorsal accessory setae of Stage IV are unchanged in Stage V.

Uropods. Inner branch: the inner branch now has from 18 to 21 plumose setae. Several hairs are found medially of the innermost setae. The fine setae occurring on the dorsal surface of the distal tip of the inner uropod in Stage IV is still found in this stage. Outer branch: the characteristic form of the outer uropod has not changed from that of Stage IV. The tip of the uropod is still drawn out as a spine and still bears thorns on its lateral margins. In this stage there are from 20 to 23 plumose setae on the posterior margins of the outer uropods. Fine, accessory setae occur ventrally between #1 and #2, #4 and #5, #9 and #10, and #14 and #15.

THE LARVAL STAGES

The five larval stages may be separated by the following key:

A. Uropods absent

1. The posterior margin of the telson has five plumose setae on each side; the third maxilliped is rudimentary. STAGE I
2. The telson has six plumose setae on each side; the third maxilliped is functional. STAGE II

B. Uropods present

1. The inner uropods are naked; pleopods are absent. STAGE III
2. The inner uropods have setae; pleopods are absent. STAGE IV
3. Pleopods are present. STAGE V

The sizes (total lengths) of the larvae in each stage were as follows (range and mean):

Stage	Minimum	Mean	Maximum	Number measured
I	2.6 mm.	2.7 mm.	2.8 mm.	8
II	3.1	3.4	3.6	8
III	4.1	4.6	5.0	7
IV	5.3	5.7	6.5	11
V	7.2	7.6	7.8	11

All the larvae measured and figured were from the Magdalena Bay area.

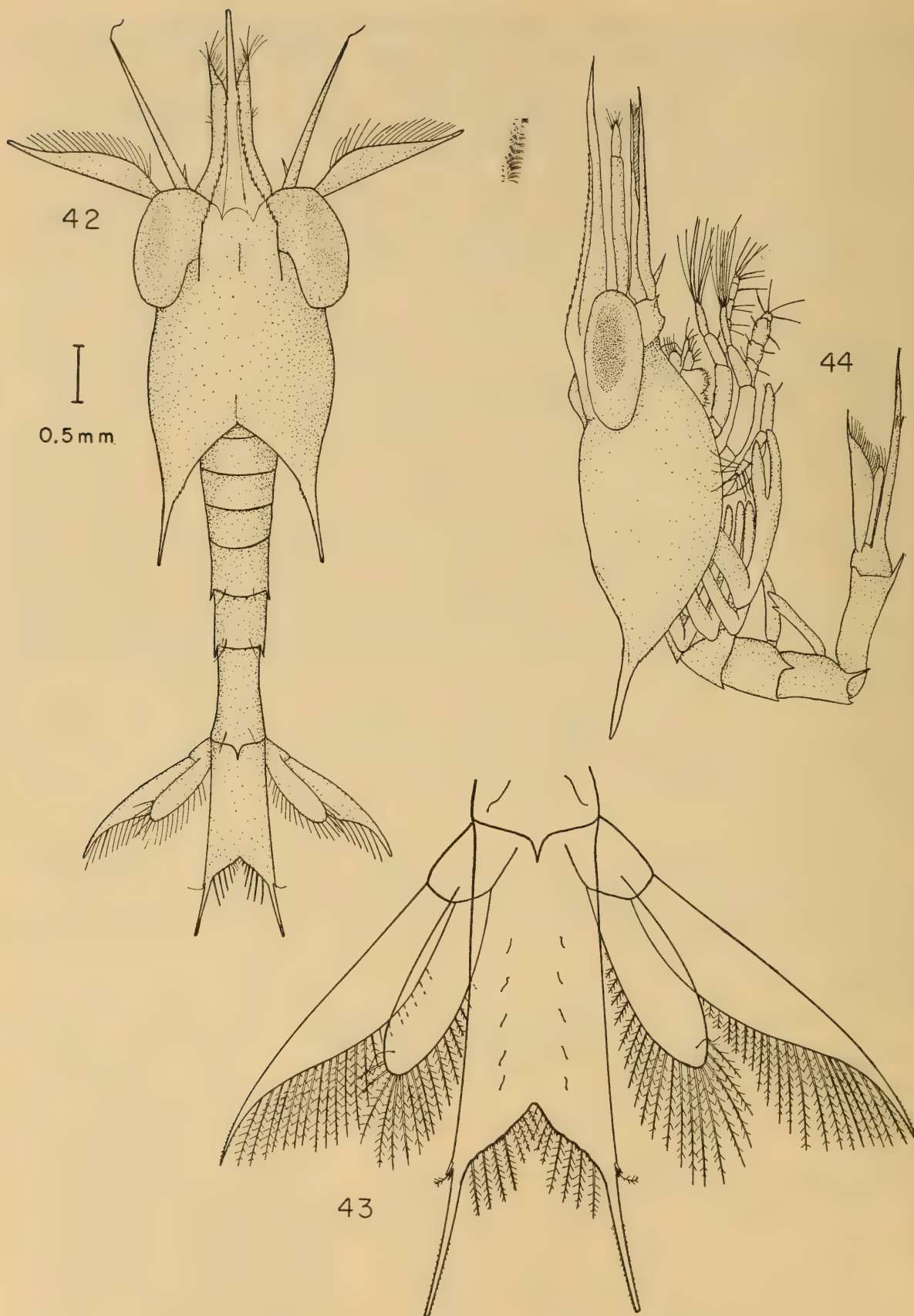


FIGURE 42. Larva, Stage V.
FIGURE 43. Telson, Stage V.
FIGURE 44. Larva, Stage V.

Lebour (1930) notes that *Galathea dispersa* may omit either Stage IV or V, but that there are usually five stages. It is not known if all *P. planipes* larvae pass through each of the five stages before molting to the juvenile stage; one or more stages may be by-passed. However, the measurements given above indicate that this is infrequent.

DISCUSSION

All except one of the developmental features of *Munida* and *Galathea* larvae as described by Lebour (1930; p. 177) are also found in *P. planipes*: the pleopods do not appear as small knobs in the third stage in *P. planipes*, but appear without previous indication in the fifth stage. *Pleuroncodes* larvae share the following traits with larvae of *Munida*, but not with those of *Galathea*: the endopod of the first maxilla has only one segment; in Stages I and II the lateral-most spine of the telson of both *Munida* and *Pleuroncodes* is longer than any of the posterior margin on the telson; a large dorsal spine occurs on the posterior margin of the sixth abdominal segment in Stages III, IV and V. *Pleuroncodes* thus appears to be more closely related to *Munida* than to *Galathea*.

The larval stages of five species of the genus *Munida* have been described. G. O. Sars described the larvae of *M. sarsi* Brinkman in 1889. Johan Huus (1934) found that these were morphologically identical with the larvae of *M. tenuimana* M. Sars, with the exception of some minute hairs in the medial cleft of the telson. Lebour (1930) described the larval stages of *M. rugosa* (Fabricius) and later (1931) set up a key to distinguish the galatheid larvae of the Plymouth area. *M. rugosa* is, however, the only species of the genus *Munida* covered by this key. (Lebour described the larvae as *M. banffica*; Zariquiey (1952), however, has synonymized this species with *M. rugosa*.) Rayner (1935) described five larval stages of *M. gregaria* (Fabricius) and *M. subrugosa* (White) but was unable to distinguish the larvae of the two species. The adults of these two species are limited to the southern hemisphere and along the Eastern Pacific they do not occur north of 41° 30' S. (Matthews, 1932; p. 470).

Larvae of *Pleuroncodes planipes* can be easily distinguished from the described larvae of European munids by the aciculate second antennae and the elongated postero-lateral spines of the carapace of the latter. They are, however, very much like the larvae of the New World munids described by Rayner (1935). The chief difference is the lack of serration on the lateral margins of the rostrum of the larvae of *M. gregaria* and *M. subrugosa*. The rostrum of each of the stages of *P. planipes*, except the first, is strongly serrated. The fifth stage of the *Munida* species has some serration on its rostrum, but not to the degree found in *P. planipes*. Rostral serration probably will not hold good in the future as a valid characteristic setting off the genus *Pleuroncodes*, for Gurney (1942; p. 255) mentions an unidentified galatheid larva from Bermuda which had a serrated rostrum. No species of *Pleuroncodes* is known from that area.

Both Huus (1934) and Lebour (1930, 1931) use color and arrangement of pigmented areas as an aid to specific identification. All color fades, however, from specimens kept longer than a few weeks in either alcohol or formalin. No color pattern was noted in *P. planipes* for that reason.

The area off Magdalena Bay (24° 40' N., 112° W.) appears to be peculiar as far as *P. planipes* larvae are concerned. Within a 50-mile radius of this bay,

densities up to 42,000/1000 m.³ have been observed and are not uncommon. Outside this area, but well within the usual distributional area of adult *P. planipes*, the larvae, as sampled with a meter net from 140 m. to the surface, have an average density of 5/1000 m.³ (based on 22 tows taken between January and July; range 0–26/1000 m.³). On this evidence it would appear that the Magdalena Bay area is a center of breeding. This is somewhat unusual in that adults are considered epi-pelagic and are distributed over a very wide range, from about 16° N. to 36° N. and from the littoral to several hundred miles off-shore.

The author wants to express his thanks to Dr. Martin W. Johnson for the help he has given me in this study. The author is also grateful for the assistance of Dr. E. W. Fager and Mrs. Margaret K. Riedel in criticism and preparation of this paper.

SUMMARY

The larval stages of a galatheid decapod crustacean, *Pleuroncodes planipes*, have been identified, described and figured. There are five stages. First stage larvae were hatched from eggs carried by adults; no larvae molted under laboratory conditions. Larval morphology indicates that the genus *Pleuroncodes* is more closely allied to the genus *Munida* than to *Galathea*. The larvae occur in high densities (42,000/1000 m.³) in the region of Magdalena Bay, Baja California, Mexico.

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THE MOULTING CYCLE IN *BALANUS BALANOIDES* L.

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Darwin, as early as 1854, noticed that a number of operculate cirripedes often moulted their skin, which included the exuviae of prosoma, appendages, penis, oesophagus, rectum and the inner lining of the mantle. He also stated that the frequency of moulting was related to the speed at which the barnacles grew.

Recently Costlow and Bookhout (1953, 1956) studied the frequency of moulting in relation to growth in recently settled cyprids of *Balanus improvisus* and *Balanus amphitrite*, but, since their observations were limited to a certain length of time after settlement, no accurate and detailed information is available regarding the seasonal fluctuations in the moulting rhythm and the factors controlling it.

The following investigation was therefore carried out to study the moulting and the factors influencing it in the boreo-arctic species *Balanus balanoides* L.

METHOD

Suitable groups of *Balanus balanoides* L. were obtained for the investigation on the shells of living *Mytilus edulis* growing on the piles of Bangor Pier in the Menai Straits, North Wales. In such a position, with a good current of water flowing past, both mussels and barnacles obtain abundant food and grow well. As soon as possible after collection all barnacles other than *B. balanoides* were removed. Usually 10–20 mature individuals were left on each shell. Care was taken not to perforate the shells of any specimens which were to be used in the experiment, since even a slight puncture, which can readily be made at the basal attachment, may lead to the death of the individual in a few days. During the first few days individuals which died were removed from the group and were not considered in the experiment.

Each group, comprising 20–30 individuals, was kept in 6-inch diameter glass crystallising dishes and the water was changed every day. It was not convenient to simulate the semi-diurnal tide; however, it was found beneficial for the barnacles to be out of water for about 8 hours each day. The dishes therefore were filled at night and emptied each morning and the cast skins present were counted. Sometimes during the breeding season pools of sperms were seen, probably indicating that copulation had occurred. Occasionally, during the period of liberation, swarms of nauplii were noted. However, if liberation occurred soon after dark, the larvae did not swarm to the side of the dish but were often devoured by the parents by the following morning. Large numbers of chocolate-red faecal pellets, containing much larval pigment and undigested chitinous appendages, were produced in consequence of this cannibalistic meal. These served to indicate that liberation had taken place. The presence of sperm pools and the evidence of liberation of nauplii were carefully noted whenever they occurred.

In some of the experiments the barnacles were fed liberally on *Artemia* larvae, and in others no food was given, a change of water being made only once a day. Both the fed and the starved animals were maintained at a series of temperatures. One series was kept in a thermostatically controlled refrigerator at $3 \pm 1^\circ \text{C}$., a second in a thermostatically controlled cool cabinet at 5 to 7°C ., a third set in a cool basement, the temperature of which differed by only 1 – 2°C . from that of the mean sea water temperature, and a fourth in a laboratory where the temperature remained considerably higher, ranging between 15 – 21°C .

In order to study the influence of the parasite *Hemioniscus balani* on the moulting rhythm, infected specimens of *B. balanoides* L. and of *Elminius modestus* Darwin were collected from Brixham, S. Devon, where a high level of infection occurs (Southward and Crisp, 1954). Infected specimens of *B. amphitrite* var. *denticulata* Broch and *B. perforatus* Bruguière were collected from the warm water docks at Swansea and from the coast of the Gower Peninsula, respectively. For these experiments animals were isolated in dishes and fed on *Artemia* larvae; subsequent examination revealed whether they were infected.

SEASONAL VARIATION IN THE MOULTING RHYTHM

Darwin (1854) in his monograph on Cirripedia mentioned that he was informed by a Mr. Peach that off the coast of Cornwall barnacle exuviae were most abundant in April–May and again in September. The specimens sent to Darwin included

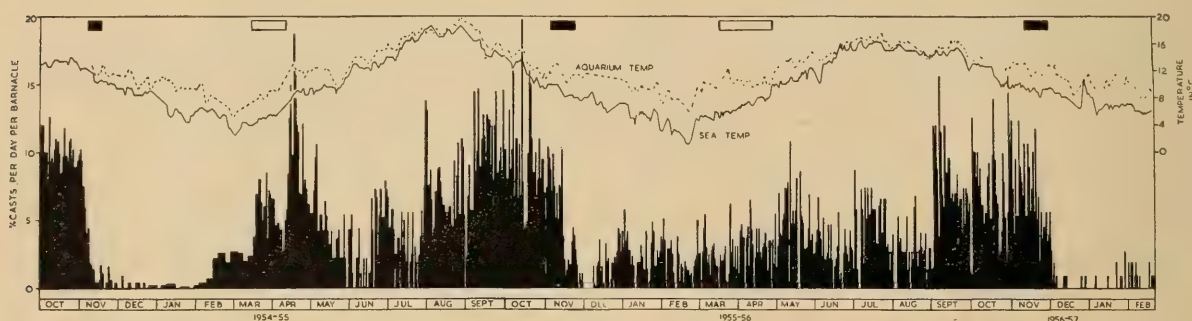


FIGURE 1. Seasonal variation in the moulting rhythm of groups of *B. balanoides* L. collected at approximately fortnightly intervals during the years 1954–57. Periods when fertilisation is taking place are shown by dark rectangles, and periods when liberation takes place are shown by clear rectangles.

B. balanoides L., *B. perforatus* Bruguière and *Chthamalus stellatus* Poli, but it is difficult to say to which species these exuviae belonged. Darwin also quotes Mr. Thomson's observation that 20 specimens of *B. balanoides* kept alive for 12 days shed 21 casts; presumably one must have moulted twice and the rest once. However, no information is given regarding the condition under which they were maintained.

The seasonal differences in the frequency of moulting of *B. balanoides* over a period of two successive years were determined from the moulting frequency of groups collected at short intervals, roughly once a fortnight, after which a new batch was substituted for the previous batch. It was found unnecessary to feed this species artificially, since for these short periods the moulting rate was not

appreciably influenced (see below and Table III). The moulting rates of such groups are presented in Figure 1 as per cent casts per day, together with temperatures both of the sea and of the cool basement where the groups were kept for observation.

It will be clearly seen from Figure 1 that the rate of moulting, which was about 8–12% per day in September–October, fell dramatically by November–December after the natural population had become fertilised. During December–January animals almost ceased to moult. The period of anecdyasis before the animals resumed moulting varied slightly with the individual and its environment. Thereafter the moulting rate increased gradually from 3–5% per day during late February–March, to a maximum value of 8–12% per day by late April–May. However, it dropped slightly by June–August, but increased thereafter till breeding activity started.

The seasonal variation of temperature did not correspond in any way with changes in the frequency of moulting, for, though the temperature remained fairly high during summer, the frequency of moulting in both years diminished in mid-summer and rose again by September and October.

LOSS OF PENIS

Crisp and Patel (1958) reported that in *B. balanoides* only, the first cast after the period of anecdyasis contained all tissues of the penis, separated by an abscission layer of new cuticle, and that this phenomenon took place whether the particular animal had been fertilised or not. Figure 2 shows the appearance of the cast skin of this species at different times of the year and the opaque appearance of the penis (Fig. 2b) in the first cast after the end of anecdyasis is due to the tissues remaining in it.

During the year 1957–58 measurements of the size of the penes of from 10 to 15 cast skins were taken every day. The individuals were of the same approximate age group, mostly exceeding two years; no young individuals were used. During the period of anecdyasis, measurements of penis length were made from individuals removed from the experiment. The results are shown as a series of mean values in Figure 3. A new penis gradually developed during the period of summer growth, reaching its maximum length before the onset of the next breeding season (Fig. 2a), and was subsequently lost after the period of anecdyasis as described above.

Figure 4 illustrates the moulting behaviour after fertilisation had occurred and shows that all individuals gave out this particular type of cast skin once, and once only, after anecdyasis; subsequent moults were normal, a stump being left in the position of the penis (Fig. 2c).

In order to confirm that the animals did not give off their penes as a result of laboratory conditions, a number of specimens were collected from the shore during the second week of January, 1957, and the penis length measured. Figure 5 shows the distribution of penis length throughout the population. The results show a clear bimodality on account of there being two types of individuals, those which had lost and those which had retained the penis. By distinguishing between those which had lost and those which had retained the penis it was possible to

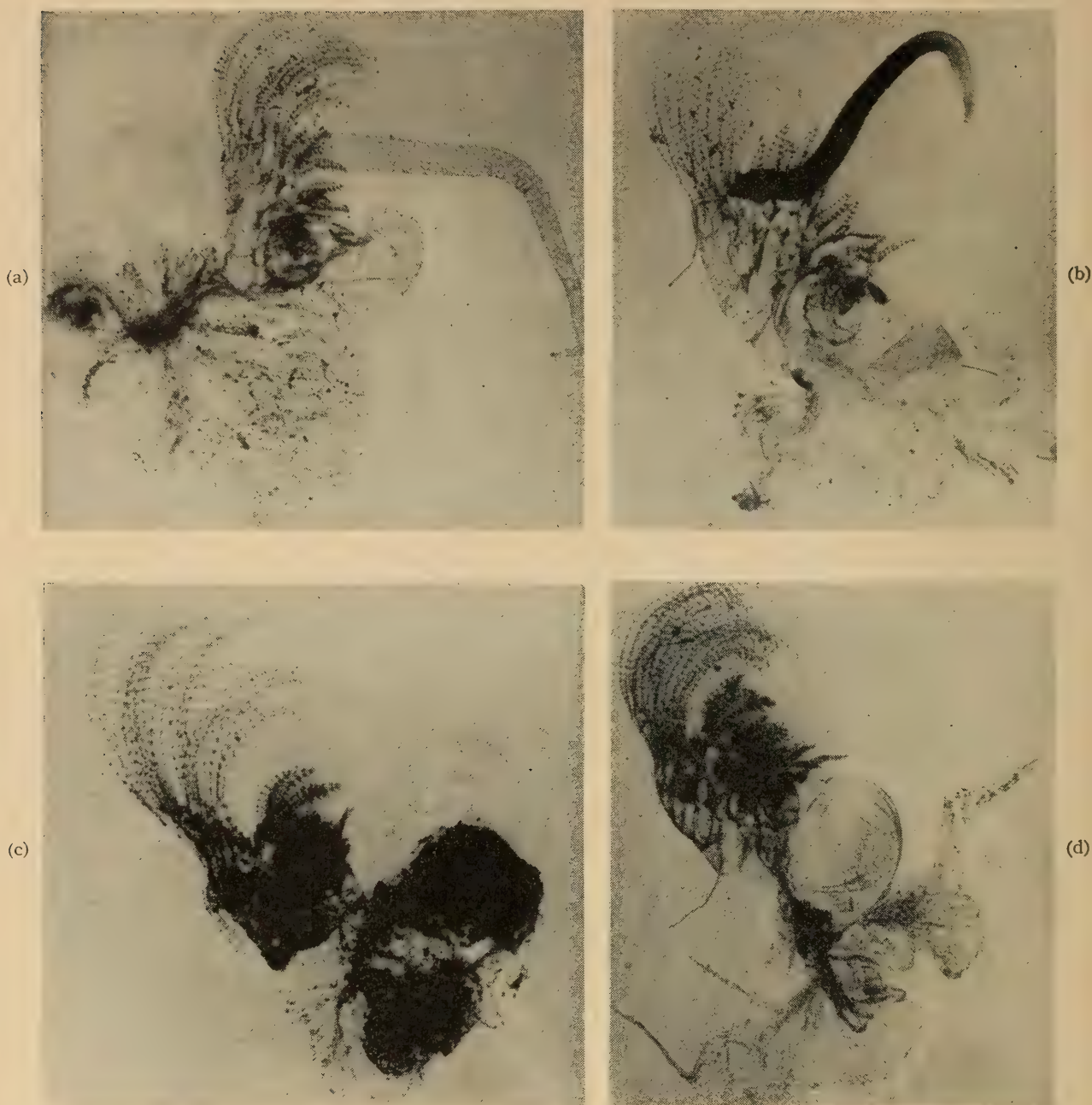


FIGURE 2. Photographs of the cast skins at different times during the year, showing the seasonal changes in appearance of the penis. (a) September–November: Cast with the skin of fully developed penis. (b) January–February: First cast after the period of anecdyosis with all tissues of the penis shed in the penis skin, rendering it opaque. (c) March–April: Post liberation cast with remains of egg masses, scarcely any penis present. (d) May–August: Summer cast with developing penis stump.

show that the loss of penes occurred earlier in barnacles growing at high water mark than those growing at mean tide level and low water mark (Table I). This appeared to be due to the fact that breeding activity started first in those settled high up on the shore (Crisp, 1959) and which were therefore correspondingly in advance of those lower down in casting the penis.

To investigate whether fertilisation was a necessary prelude to the loss of the

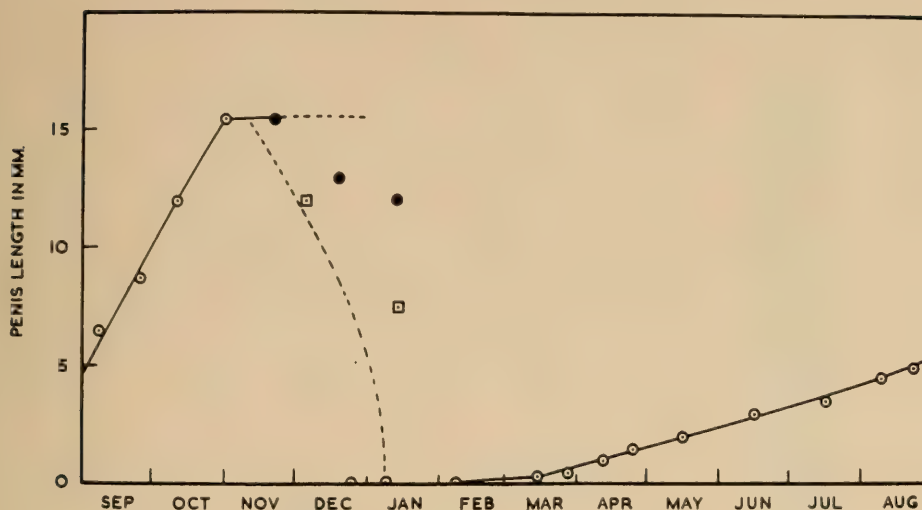


FIGURE 3. Seasonal changes in the development of the penis, measured from the cast skins. $\square$, mean values from a highly bimodal distribution of lengths due to some individuals having shed the penis. The mean size of those retaining the penis is given thus $\bullet$. Note the shrinkage in size after fertilisation, and eventual loss of the organ.

penis, mature animals collected at the end of October and early November were isolated in dishes to prevent the occurrence of copulation and fertilisation of eggs. They were maintained at temperatures corresponding closely with those in the sea. Not only the individuals which already had fertilised egg masses, but also

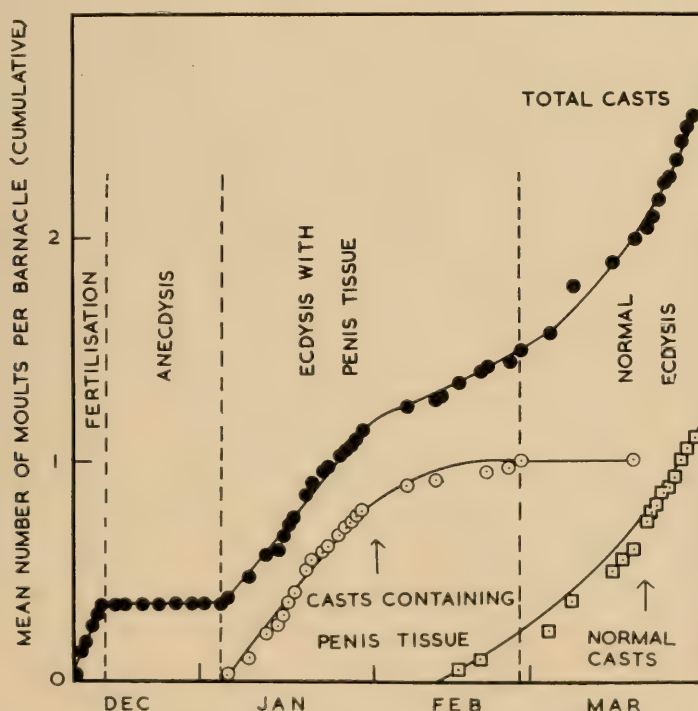


FIGURE 4. Moulting rate of 36 specimens of *B. balanoides* L. at 7° C. between 8.XII.1957 and 29.III.1958 showing how, after the period of anecdyosis, the first moult contains all tissues of the penis and the subsequent moults are normal. $\bullet$, total number of all casts shed. $\circ$, casts containing penis tissue, shed in January and February, one only per barnacle. $\square$, normal casts shed after the middle of February.

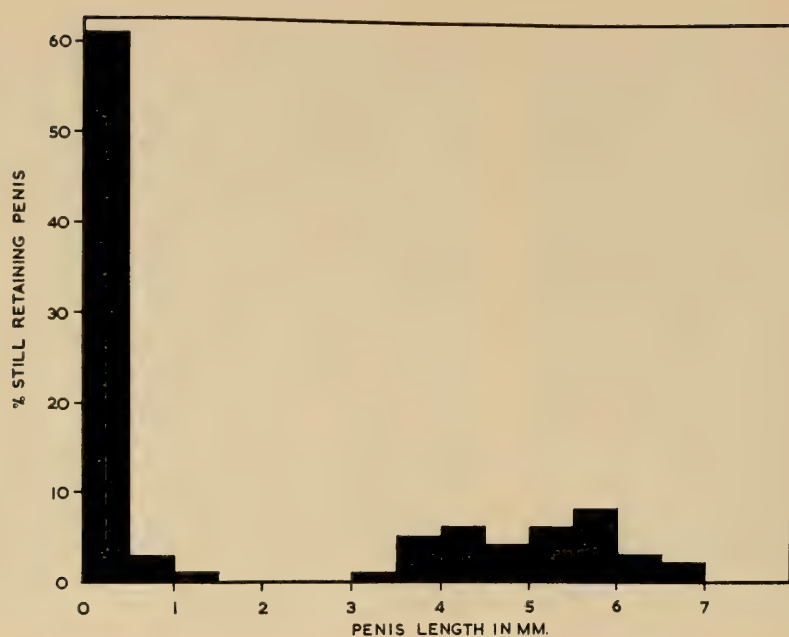


FIGURE 5. Distribution of penis lengths in a natural population of about the same year group on January 11th, 1957, showing clear bimodality on account of there being two types of individuals, those having lost and those still retaining the penis.

those which were still unfertilised gave off the cast with penis tissues (Table II). This indicates that fertilisation of itself had no marked effect on the loss of the penis.

B. balanoides could also be prevented from becoming fertilised by keeping them at fairly high temperatures (15° – 21° C.) throughout the summer and autumn. These unfertilised animals continued to retain a normal penis, but if they were subsequently transferred to a temperature of 6° C. and kept cool for several weeks, they entered the breeding condition, became fertilised, and showed the typical pattern of anecdyosis and loss of penis. Others were maintained at 5° to 7° C. throughout the summer and autumn, but, on account of abnormal laboratory conditions not fully understood, reached maturity considerably later than those kept in the cool basement or those growing normally in the field. These animals, although kept in the cold for a long period, retained a normal penis until breeding had taken place. After having bred, all individuals gave off their penis with the first cast following anecdyosis. Furthermore, in some experiments one set of barnacles were starved and another fed; this did not influence in any way the loss of the penis.

It is clear therefore that the loss of the penis is not directly dependent on fertilisation nor on maintaining the animals at low temperature, nor on the availability of food. It appears to be a part of a normal physiological cycle, in which the gonads as a whole undergo recession, the loss of the penis being accompanied by

TABLE I

Percentage of fertilised population, collected from different tide levels, which had still retained the penis on January 11th, 1957

Level	% with penes
H. W.	10.5
M. T.	20.4
L. W.	37.8

TABLE II

Percentage of animals which lost the penis after the period of anecdysis when maintained at 7° to 12° C.

Date animals were removed for examination	Number of specimens used		Percentage which gave off casts containing penis tissues	
	Animals without egg masses	Animals with egg masses	Animals without egg masses	Animals with egg masses
9.I.1958	188	25	42.5	28.0
11.I.1957	14	12	50.0	50.0
21.II.1958	30	36	97.0	94.0

shrivelling of the vesiculae seminalis and degeneration of testes. This takes place only after they have experienced such conditions as allow the gonads to reach their full development, and under normal circumstances for them to become fertilised.

This phenomenon was observed only in this species. Crisp (1954), however, had reported in *Balanus porcatus* (da Costa) a shortening in the length of the penis after fertilisation, followed by gradual lengthening during the summer, reaching a maximum length just before copulation.

MOULTING RATE OF YOUNG BARNACLES

Costlow and Bookhout (1953, 1956) studied the moulting rate of recently settled cyprids of *Balanus amphitrite niveus* and *B. improvisus* under varying conditions of light and food till they reached the adult stage.

During the present investigation the moulting rate of recently settled spat of *B. balanoides* was studied.

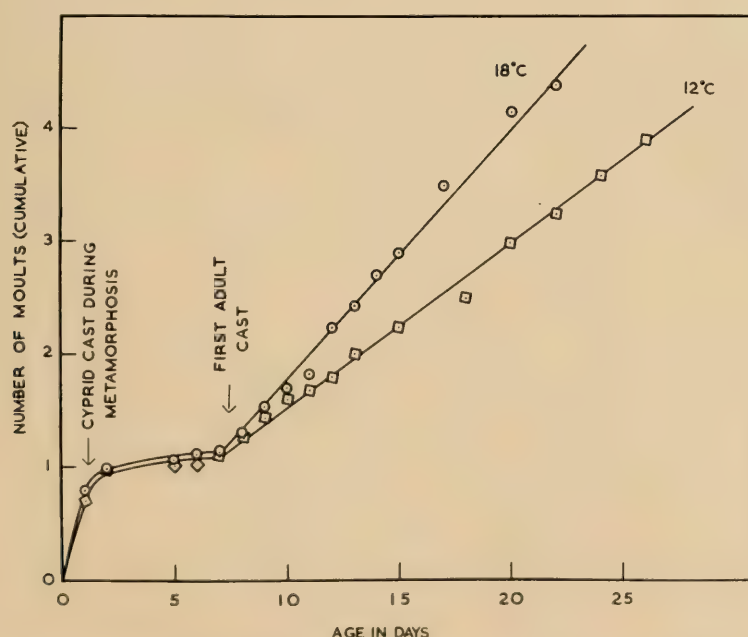


FIGURE 6. Moulting rate of young spat of *B. balanoides* L. of known age when kept at 12° C. and 18° C.

Cyprids which had settled on inert plastic plates and metamorphosed were brought into the laboratory and kept in dishes. The water in the dishes was stirred mechanically and *Chlamydomonas* sp. were offered every day as food. Since satisfactory conditions could not be guaranteed in the laboratory, these plates were returned to the sea. At the same time another batch, which had been put out for settlement, was brought into the laboratory, and was placed in a dish; the water was stirred and the animals fed as before. Several plates were thus kept in rotation between the laboratory and the sea, a few plates being kept continuously in the laboratory. The number of exuviae was recorded each day.

Figure 6, drawn from the sets of results, illustrates the cumulative number of casts shed during the first few weeks after settlement. Metamorphosis of cyprids occurred after 24 to 36 hours and the apodemes of the paired eyes, the bivalve carapace and the exuviae of the thoracic limbs were shed (Crisp and Stubbings, 1957). No further ecdysis occurred for at least 5 days. The time of the first adult cast varied considerably, but on average it took place between 9 and 10 days after metamorphosis. This was longer than the corresponding period for *B. amphitrite* (Costlow and Bookhout, 1956), in which the first cast occurred between 3 and 5 days after metamorphosis. Thereafter *B. balanoides* moulted approximately once every four days at 18° C. and about once every six days at 12° C. From Figure 6 it will be clearly seen that the moulting rate of the rapidly growing spat was at least double the maximum rate of moulting of the adult over the same period of the year (Fig. 1).

FACTORS INFLUENCING ECDYSIS

a. Feeding

Groups of *B. balanoides* were maintained at several temperatures, some being fed on *Artemia* larvae and other groups having the water changed only. Those groups which were given *Artemia* were observed to be beating the cirri intermittently throughout the day and producing dark red faeces. The starved groups, on the other hand, were active for a short time after the water in the dishes had

TABLE III

*Moulting rate of B. balanoides under varying conditions of temperature and feeding.
The animals were collected on July 1st, 1957*

Number of animals available	Condition		Rate of moulting	
	Fed or starved	Mean temperature	First 10 days after collection	First 60 days after collection
46	fed	19.5° C.	0.10 casts per day	0.13 casts per day
47	starved		0.10 casts per day	0.065 casts per day
22	fed	17.0° C.	0.12 casts per day	0.12 casts per day
21	starved		0.10 casts per day	0.06 casts per day
27	fed	6.0° C.	0.05 casts per day	0.046 casts per day
29	starved		0.05 casts per day	0.032 casts per day
27	fed	3.0° C.	0.05 casts per day	0.03 casts per day
27	starved		0.05 casts per day	0.03 casts per day

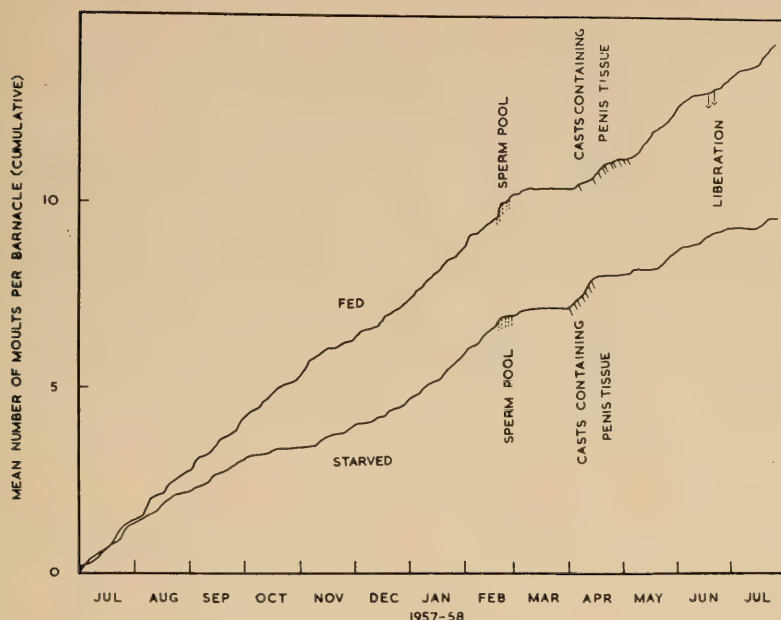


FIGURE 7. Moulting rate of groups of *B. balanoides* L. showing the effect of starvation at 5–7° C. Sperm pools indicate that fertilisation was taking place. Casts containing penis tissues are shown by oblique lines and liberation by small arrows.

been changed, but remained quiet for the rest of the day except for very occasional beating.

As can be seen from Table III, both fed and starved batches moulted at approximately the same frequency immediately after collection and for the following ten days. Thereafter the fed barnacles continued to moult at the same or at a slightly decreased rate, but the starved barnacles showed a progressive reduction in frequency but did not cease to moult altogether. These long-term changes are shown in Figure 7 for animals kept at 5 to 7° C. It will be noticed that after anecdysis the moulting rate was low, but it recovered much more rapidly in those individuals which were fed. These results suggest that the amount of reserve food available is important in determining the moulting rate. The results in Table III show that at higher temperatures there is an even more marked difference between fed and starved individuals due, no doubt, to the greater loss of reserve food at the higher metabolic rate.

b. Temperature

Southward (1955a), who studied the influence of temperature on the cirral activity of *B. balanoides*, reported that the rate of cirral beats increased linearly with the increase in temperature from 3 to 20° C. The rate of beating, however, fell with a further rise in temperature, 31 to 32° C. being lethal.

Similarly (Fig. 7) the moulting rate of barnacles fed on *Artemia* increased linearly with the rise in temperature from 3 to 20° C. The moulting rate of starved specimens, on the other hand, increased from 3 only up to 12° C. and then diminished with a further rise in temperature. If, as seems likely, the rate of moulting is influenced by the food reserves available, it would not be surprising that with increased metabolic rate at higher temperatures the reserves would fall

sufficiently to reduce the moulting rate. After a sufficient period the effect of loss of reserves might more than counteract the normal increase in moulting rate with temperatures, so that the moulting rate actually fell with rise in temperature.

Both fed and starved specimens moulted at the same frequency at the lowest temperature (Table III, Fig. 8); the reason for this might be the fact that at 3–4° C. those which were offered food remained quiet and fed very little, while those which were starved did not lose reserve metabolites at any appreciable rate at low temperatures.

Southward kept his specimens only for a very short time at 25 to 30° C. and was able to measure their cirral beat at these temperatures; however, both fed and

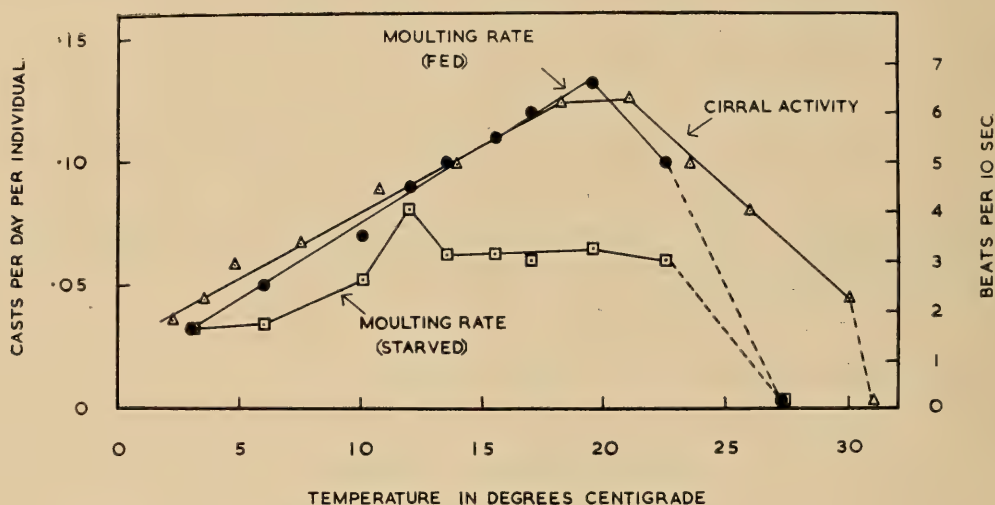


FIGURE 8. The effect of temperature and feeding on the moulting rhythm of *B. balanoides* L. and the effect of temperatures on cirral activity (from Southward, 1955a).

starved specimens during the present series of experiments died if kept for more than three days at 25 to 27° C.

c. Breeding

The majority of recently fertilised animals abruptly ceased to moult for some six to eight weeks (Fig. 9); this might be regarded as an adaptation to prevent recently oviposited eggs, which are not yet hardened, from being shed with a cast. However, after anecdysis, the still gravid barnacles resumed moulting, especially if they were fed (see Figure 7) and were found to have cast the skin with a torn-off mantle lining, due to the pressure exerted by the egg masses. The latter had by this time become hard and were pressed well up against the mantle lining of the parietes and basis. Occasionally only the exuviae of the appendages and prosoma were shed, the mantle lining being retained and shed later at the time of the liberation of the nauplii hatched out from the egg masses.

It will be seen from Figure 9 that the barnacles which were kept from being fertilised by being artificially isolated continued to moult when the fertilised population had ceased. Nevertheless they showed a sharp fall in moulting rate by the end of November. This suggests that the drop in the moulting rate may be due to a basic physiological rhythm, like that controlling the loss of the penis and

degeneration of the gonads, and that the sudden onset of this process in fertilised barnacles may be due to the stimulus of copulation or oviposition.

d. *Effect of emersion on moulting*

As Darwin (1851) observed, the skin cannot be shed except when the barnacle is immersed in water. The period of emersion of organisms which grow between low water neap and high water neap tide levels never exceeds about 12 hours. In a humid environment intertidal barnacles may be kept out of water quite healthily

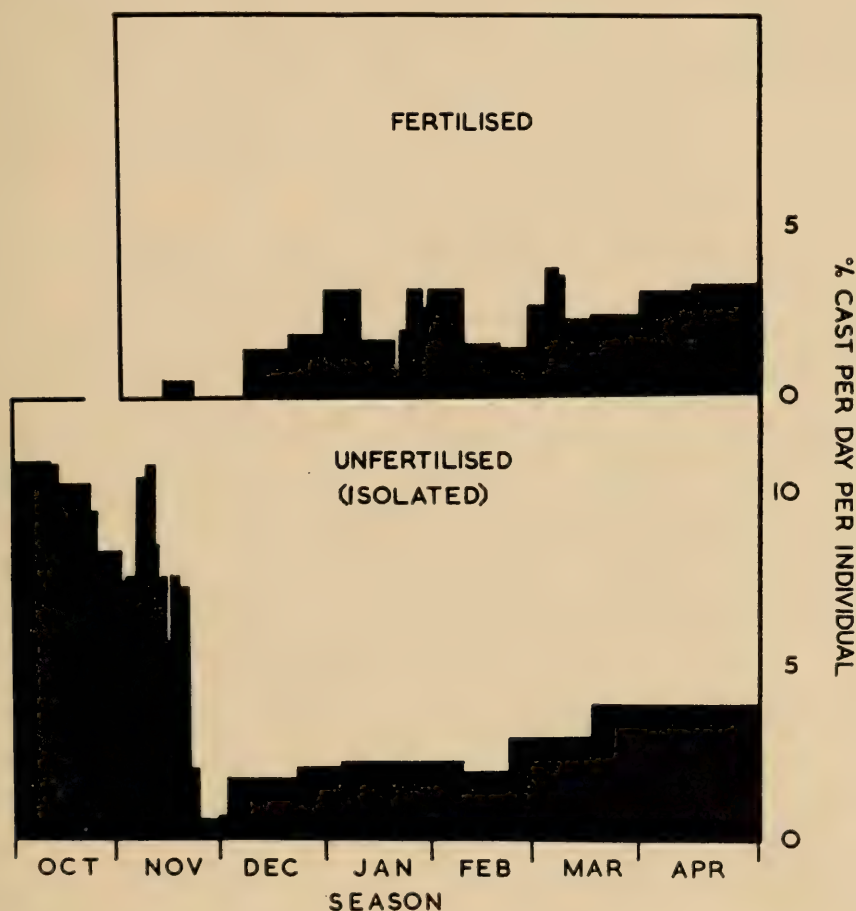


FIGURE 9. Moulting rate of isolated specimens not carrying embryos and of those with fertilised egg masses, measured over the same period. Fertilised specimens show an immediate drop to zero; unfertilised specimens continue to moult freely in mid-November, but show a fall in late November at the end of the normal breeding season.

for several days. It is thus possible to ascertain whether the frequency of moulting is influenced by an abnormally long period of emersion.

Four groups of barnacles growing under nearly identical conditions on settlement plates on a raft were brought into the laboratory. Here they were kept under a damp cloth for varying periods of time. The total number of casts shed when the barnacles were introduced for a period of 24 hours into aerated sea water was recorded. In Table IV the aggregate percentage of casts (per 100 barnacles) is shown against the total number of days kept in the laboratory. The letter "D"

TABLE IV

Moulting rate in relation to the treatment of various groups of barnacles; letter "D" indicates the days when the specimens were kept dry, on the other days they were kept in sea water at 12° C.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Group	Number of barnacles used in experiment	1st day	2nd day	3rd day	4th day	Total casts after 4th day
a	382	D	D	D	26.2%	100
b	373	D	D	22.0%	35.4%	132
c	370	D	14.3%	20.2%	28.6%	106
d	453	3.7%	D	D	33.1%	150
Mean	—	—	—	—	30.9%	(7.7 ∞ per day)

$$\chi^2 = 9.98$$

indicates the days when the groups were kept dry, and no casts were therefore emitted. Column 7 gives the total number of casts for the whole period.

It is clear that moulting proceeds steadily throughout the experiment and is not delayed significantly as a result of the barnacle being out of water. The numbers of casts found after 4 days in the different groups, a, b, c and d, which were treated differently give a value for χ^2 of 9.98 when compared with the expectation of 7.7% per day per individual. This is slightly higher than the value of $\chi^2 = 7.8$ for the 5.0% significance level. Since, however, there was no consistent trend in the moulting rate in relation to the emersion period, the significance of the χ^2 value was probably due to intrinsic variations in the rate of casting of the four groups, a, b, c and d; such variations are frequently observed between groups of individuals from apparently similar habitats. The data of Table IV, rearranged to show the moulting rate in relation to the time kept out of water, are displayed in Table V. The rates of casting are closely uniform and the χ^2 test applied to the differences was found not to be significant ($\chi^2 = 6.26$ for 3 degrees of freedom). The number of casts produced in a given time is therefore not altered significantly by emersion up to a period of four days.

TABLE V

Moulting rate in relation to the total time out of water

(1)	(2)	(3)	(4)
Number of days out of water	Barnacle days (No. of barnacles × no. of days)	Number of casts	Moulting rate (% casts per day)
4	1528	100	6.55%
3	2478	215	8.69%
2	740	53	7.17%
1	1566	120	7.68%

$$\chi^2 = 6.26$$

It was often noticed that the rate of moulting for the first day or two was higher than the average for the whole period. This may have been partly due to the individuals having been out of water since the previous high tide. Possibly also the changed conditions in the laboratory caused a temporary rise in the rate of moulting. However, this small initial rise had no significant influence on the average rate taken over two or three weeks.

e. Tidal periodicity

In many marine organisms, especially in lamellibranchs and in annelids, the effect of moon and tide on the breeding cycle had been well established (Korringa, 1947.) However, Crisp and Davies (1955) reported that *Elminius modestus* bred at any time irrespective of the tidal cycle.

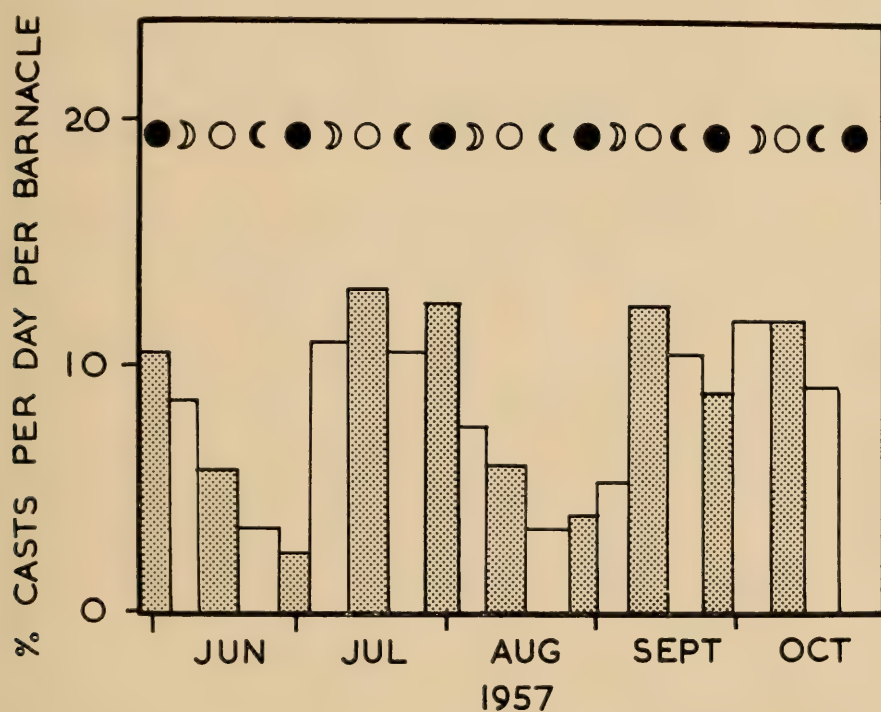


FIGURE 10. Average moulting rate of the groups of *B. balanoides* L. collected at monthly intervals and kept at sea temperature over the spring and neap tide periods (shown by moon phases). Stippled blocks, average moulting rate over the spring tide period; clear blocks, average moulting rate over the neap tide periods.

Churchill (1917-18) studied the effect of the lunar cycle on the moulting rhythm of the blue crab *Callinectes sapidus*, but on the basis of his results dismissed the popular belief prevailing that both moon and tide had a marked effect on the moulting of crabs as being a folklore superstition. However, Wheeler (1937) in *Anchistoides*, Nouvel and Nouvel (1939) in the mysid *Praunus flexuosus* and Nouvel (1945) in *Leander serratus* found that the largest numbers of exuviae were shed on the day corresponding to the largest tide. However, their observations were made only over a single lunar period and may therefore be fortuitous. Kinne (1953, 1959) found no tidal rhythm in the moulting of the amphipod *Gammarus duebeni*. Tidal rhythm in moulting does not therefore seem to be of general occurrence in the Crustacea.

During the present investigation experiments were carried out to test if any tidal rhythm could be found in barnacles. Each tidal cycle was divided into two periods, approximately midway between the largest and the smallest tides, one period corresponding to the spring and the other to the neap tides (shown by moon phases in Figure 10). The average moulting rate of groups of animals collected at monthly intervals was determined for pairs of successive neap and spring periods. The difference between the average for neap and spring periods was tested by applying Student's "t" test and found to be insignificant. Ecdysis therefore occurred at approximately regular intervals, provided barnacles were immersed, irrespective of the tidal cycle.

f. *Effect of tidal level*

Animals were collected from three different tidal levels and their moulting rate for the first ten days was recorded. It can be seen from Table VI that there was no significant difference in the moulting rhythm. Though Southward (1955b) did record specimens (*B. balanoides*) collected from low water mark beating

TABLE VI

Moulting rate of B. balanoides, collected from different tide levels, for first 10 days

Dates animals were collected	Temperature	Casts per day per individual		
		H. W.	M. T.	L. W.
22nd Jan.	8-10° C.	0.03	0.04	0.05
29th Jan.	8-10° C.	0.025	0.03	0.04
28th Feb.	8-10° C.	0.06	0.04	0.04
5th Sept.	14-16° C.	0.110	0.10	0.12

markedly faster than those collected from higher up, when he repeated the experiment on the following day with the same specimens he found that the differences had disappeared. Neither moulting nor cirral activity appears to be influenced by tidal level.

g. *Influence of light on moulting rhythm*

It has been claimed that light has an inhibitory action on the moulting rhythm of Crustacea (Nouvel, 1945). However, Costlow and Bookhout (1956) did not find any significant differences in the moulting rate of the series of young spat of *B. amphitrite niveus* reared under different conditions of illumination.

During the present series of experiments the influence of light on the moulting rhythm of adult barnacles was investigated. Groups of barnacles were collected from the shore and maintained under three different conditions. One batch was kept under a light-tight box kept in continuous darkness. A second batch was kept in a continuously illuminated, well ventilated box, illuminated by a 25-watt bulb. The third batch was exposed to natural variations of light conditions, 12 hours light during the day and 12 hours darkness at night. All were kept at the

same temperature of 10° C. No significant difference in moulting rate was found over a period of 50–60 days.

h. Influence of parasite *Hemioniscus balani*

The majority of the species of British barnacles was found to be capable of harbouring the isopod *Hemioniscus balani* in the mantle cavity, in the space normally occupied by the fertilised egg masses. Crisp (1954) found this parasite causing castration of the female gonads in *B. porcatus* (da Costa) and later a similar effect was reported in *Elminius modestus* (Crisp and Davies, 1955).

The effect of the parasite on the moulting rhythm was therefore investigated using infected *B. balanoides*, *Elminius modestus*, *Balanus perforatus* and *B. amphitrite* var. *denticulata*. It will be seen from Table VII that the presence of the parasite had no significant effect on the moulting cycle of the host; the intermoult periods of infected and uninfected barnacles were not significantly different. However, as with fertilised individuals which had eggs in the mantle space, the speci-

TABLE VII

Frequency of moulting of specimens infected with Hemioniscus balani and of uninfected specimens collected from the same locality

Species	Temperature	Number of animals used in experiment	Percentage infected with <i>Hemioniscus balani</i>	Mean intermoult period in days of	
				Individuals with <i>Hemioniscus</i>	Individuals not infected
<i>Balanus balanoides</i>	12° ± 2° C.	35	57%	10.9	9.0
<i>Elminius modestus</i>	12° ± 2° C.	10	50%	7.3	7.3
<i>Balanus perforatus</i>	13° ± 2° C.	20	5%	6.0	6.4
<i>Balanus amphitrite</i>	15° ± 2° C.	100	6%	10.3	9.8

mens harbouring the parasite were found to shed exuviae with torn-off mantle linings. On no occasion was the parasite rejected with the cast, except in the large barnacle *B. perforatus*. This species was observed to reject the parasite enclosed within the casts, and this may account for the normally very low degree of parasitism present compared with that found in other species in the same vicinity.

SUMMARY

1. The seasonal variations in the frequency of moulting of boreo-arctic species of the operculate barnacle *Balanus balanoides* L. were studied during the years 1954–57 by observing groups of barnacles kept in the laboratory at temperatures corresponding to that of sea water. The rate of moulting, which was about 8 to 12% casts per day per barnacle during October–November, fell sharply by November–December and fertilised animals underwent a period of anecdyosis for about 6 to 8 weeks. They resumed moulting at a slower rate, reaching a maximum by May with a slight decrease in June–August, rising again towards the breeding season in November.

2. In this species the first cast after the period of anecdyasis always contained all tissues of the penis, separated by an abscission layer of new cuticle. Neither food, temperature nor the act of fertilisation were directly responsible for the loss of the penis. The evidence indicates that the loss of the penis is part of a physiological cycle in which gonads undergo recession after they have reached full development. A new penis gradually developed during the period of summer growth, reaching its maximum length before the onset of the breeding season.

3. Feeding influenced the moulting rhythm, but the effect made itself felt only after the first 10 to 15 days following collection from the shore. The moulting rate of specimens maintained without food for a period longer than this (*i.e.*, for about 30 to 60 days) fell considerably from the initial value, but animals fed on *Artemia* larvae continued to moult at about the same rate as when freshly brought in from the shore.

4. The moulting rate of specimens given food increased linearly with the rise in temperature from 3–20° C.; on the other hand the moulting rate of starved specimens only increased from 3–12° C. and fell considerably with further rise. Temperatures higher than 25° C. were lethal to both groups.

5. Neither the lunar cycle nor the tidal level had any influence on the moulting rhythm.

6. The parasite *Hemioniscus balani*, which caused castration of the female gonads, did not influence the frequency of moulting.

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EFFECTS OF TURBIDITY-PRODUCING MATERIALS IN SEA
WATER ON EGGS AND LARVAE OF THE CLAM
(*VENUS (MERCENARIA) MERCENARIA*)

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The effect of suspended materials on mollusks and on the survival and growth of their larvae is of interest to biologists and of considerable importance to shellfish producers. Since several of the most important commercial species are essentially inhabitants of the shallow waters of bays and estuaries, almost all of the grounds used for the cultivation of shellfish are frequently subject to water rendered turbid by natural phenomena, such as floods and storms, and by human operations, such as dredging, bridge- and road-building, etc.

An extensive field study by Lunz (1938) during the dredging of the Intra-coastal Waterway of South Carolina showed that the mortality of adult oysters in the dredged area, except where oysters were actually buried by the spoil, was no higher than in areas remote from the dredging operations. Moreover, he found no evidence that the physiological condition of the oysters, as judged by the yield of meats per bushel of oysters, was affected by the dredging operations. He also reported that the intensity of setting of oysters in an area adjacent to dredging operations did not differ from setting intensity in areas remote from such operations, and concluded (p. 134) that "dredging operations apparently had no effect on spawning and setting."

In careful quantitative experiments Loosanoff and Tommers (1948) have shown that as little as 0.1 gram per liter of silt reduced the average pumping rate of adult oysters by 57 per cent. At a concentration of 1.0 g./l. they found the reduction in average pumping rate was more than 80 per cent and reached 94 per cent when the concentrations of silt were increased to 3.0 and 4.0 g./l. They reported that results with kaolin and chalk were similar to those obtained with silt, and that 0.5 g./l. of Fuller's earth, the only concentration tested, reduced the rate of pumping by 60 per cent.

Since the development of techniques for rearing bivalve larvae in the laboratory (Loosanoff and Davis, 1950), these techniques have been used to determine quantitatively the effects of various factors, such as temperature, species of food organisms, crowding, quantity of foods and salinity on the survival and growth of bivalve larvae (Loosanoff, Miller and Smith, 1951; Loosanoff and Davis, 1953; Loosanoff, Davis and Chanley, 1953a; 1953b; Davis, 1953; Davis and Guillard, 1958; Davis, 1958). In the present study they have been employed to determine quantitatively the effect of various concentrations of several materials suspended in sea water on the development of eggs of the hard clam, *Venus (Mercenaria) mercenaria*, and on the survival and growth of their larvae.

The turbidity-producing materials used in these experiments were clay (kaolin N.F. VII Mallinckrodt), Fuller's earth (dusting powder, McKesson), chalk (pre-

cipitated chalk U.S.P. McKesson and Robbins) and silt. The silt was collected from the bed of the Wepawaug River, just upstream from our laboratory. The silt was washed through a 325-mesh, stainless steel screen (meshes approximately 50 microns square) to remove larger particles, collected in a Büchner funnel, where it was washed with distilled water to remove salt, and then dried at 200.0° C. Finally, the dried cake of silt was ground in a jar mill. In the later experiments the Fuller's earth was also ground in the jar mill. Concentrations of suspended material are, therefore, all expressed as grams of dry powder per liter.



FIGURE 1. Turbidity apparatus showing the wheel and method of attachment of bottles. Motor and reduction gear box indistinctly seen in background.

In making up a suspension, a weighed quantity of the powdered material was thoroughly mixed with sea water and the various experimental concentrations prepared by serial dilution.

The method for obtaining fertilized clam eggs in midwinter has previously been described (Loosanoff and Davis, 1950) as have the methods for determining the percentage of eggs developing to the straight hinge stage, and for determining the rate of growth of larvae (Davis, 1953, 1958).

In the present series of experiments, wide-mouthed, 32-ounce polyethylene bottles with screw caps were used as containers for the larval cultures. To keep

the materials in suspension we used a modification of an apparatus which V. L. Loosanoff of this laboratory observed in P. R. Walne's laboratory at the Fisheries Experiment Station, Conway, England. The bottles were mounted on a vertical wheel which, as it rotated, turned the bottles end over end once for each revolution of the wheel. In our apparatus provision is made for 12 bottles on each side of the wheel for a total of 24 bottles so that 12 sets of duplicate cultures can be run concurrently (Fig. 1). The wheel is kept rotating by a constant speed motor working through a speed reducer giving eight revolutions of the wheel per minute. The wheel and attached bottles are enclosed in a plywood box in which the air

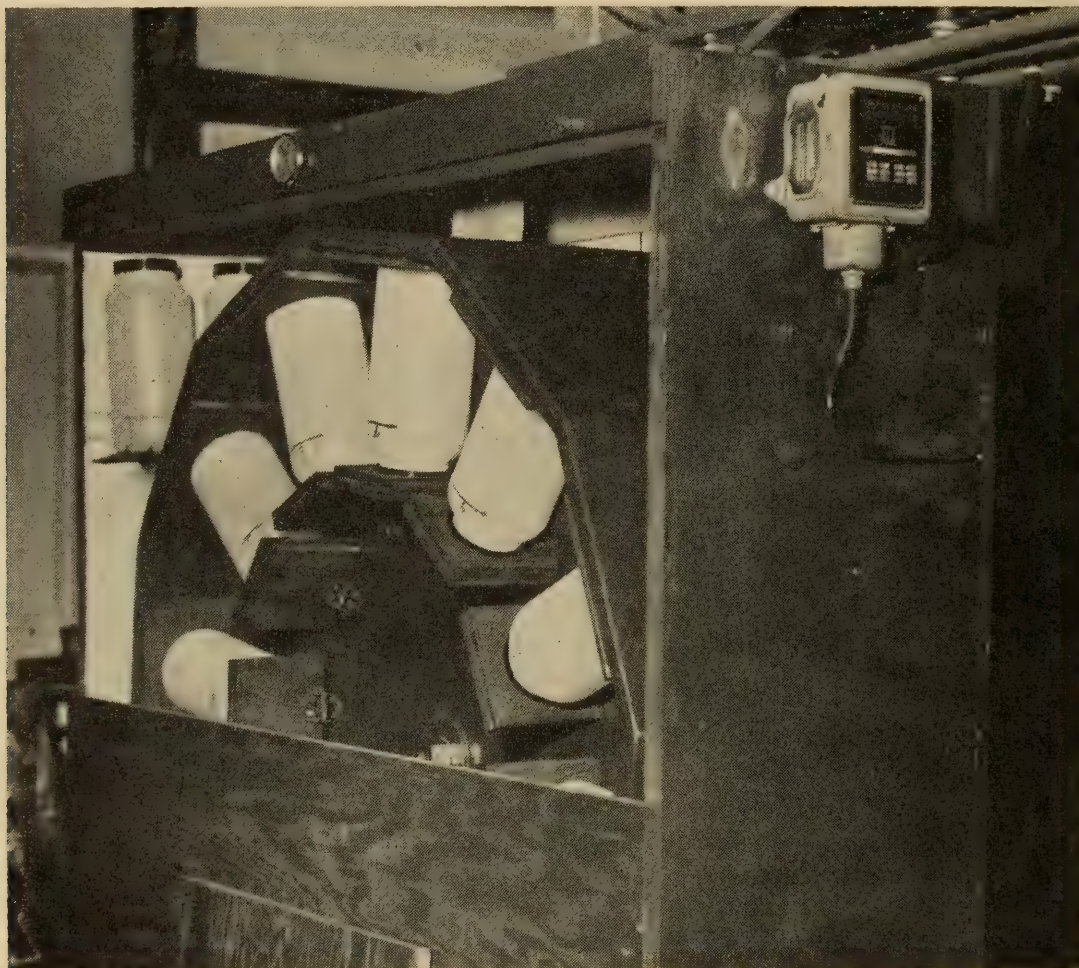


FIGURE 2. Turbidity apparatus showing thermostat control, temperature box and position of stationary control cultures.

temperature is thermostatically controlled at 24.0° C., using two 60-watt light bulbs as heaters. A wire mesh shelf is installed in the left upper corner of this box to hold two additional bottles for stationary control cultures (Fig. 2). In practice, one pair of bottles on the wheel is used for moving control cultures, leaving 11 pairs of bottles for duplicate cultures of larvae at each of 11 different sets of experimental concentrations of suspended materials.

The number of larvae suspended in the 800 ml. of sea water and turbidity-producing material in each bottle was initially the same for all cultures in an

experiment, but ranged from 8000 per bottle in some experiments to about 15,000 in others. The larvae were fed daily, each culture receiving an equal quantity of a mixture of *Isochrysis* and *Monochrysis*. The larvae were examined every second day when the sea water and its suspended material were changed, but quantitative samples for counts and measurements were taken only when the larvae were 48 hours old and on the twelfth day, when an experiment was terminated.

Preliminary experiments indicated that some fertilized clam eggs could develop into straight hinge larvae in concentrations of either clay (kaolin) or chalk as high as 4.0 g./l., but that none developed to the straight hinge stage in equivalent concentrations of either Fuller's earth or silt. Subsequent experiments, however,

TABLE I

Percentage of clam eggs developing to the straight hinge larval stage in different concentrations of suspended materials. The number of eggs developing to the straight hinge stage in the stationary controls is considered 100 per cent

Concentration g./l.	Percentages			
	Silt	Clay (kaolin)	Fuller's earth	Chalk
Stationary control	100	100	100	100
Moving control	91			
0.125	95	82	75	
0.188	90			
0.250	96	82	61	approx. 45
0.375	93			
0.500	99	52	41	
0.750	92			
1.000	79	37	57	approx. 30
1.500	65			
2.000	39*	49	50	approx. 39
3.000	0			
4.000	0	42**	45***	approx. 45****

* Averaged 195.80 μ
 ** Averaged 203.90 μ
 *** Averaged 199.60 μ
 **** Averaged 189.10 μ

At 12 days when kept in these turbidities only during the first 48 hours after fertilization and then returned to normal sea water.

have shown that if Fuller's earth was first ground in a jar mill, some larvae developed normally at 4.0 g./l. Similarly, ground silt at concentrations of 3.0 or 4.0 g./l. still completely prevented normal development of clam eggs (Table I). In silt concentrations of 0.75 g./l. or lower, however, there were no significant differences in the percentage of clam eggs developing normally, while with clay, chalk or Fuller's earth there appeared to be a more or less consistent stepwise reduction in the percentage of eggs developing normally with each increase in the quantity of suspended material (Table I).

Although in a silt concentration of 2.0 g./l. only 39 per cent of the eggs developed to the straight hinge larval stage, almost all of these larvae were capable of surviving and growing to metamorphosis (mean length 195 μ at 12 days) if

returned to normal sea water. Similarly, larvae developing to straight hinge stage in concentrations of 4.0 g./l. of clay, chalk or Fuller's earth have been reared to setting stage after being returned to normal sea water at the end of 48 hours.

Experiments on the effect of suspended materials on the growth of clam larvae that have developed to the straight hinge stage in our usual sea water have shown that such larvae cannot grow in concentrations of clay, Fuller's earth or chalk as high as those at which some eggs developed. For example, there was no evi-

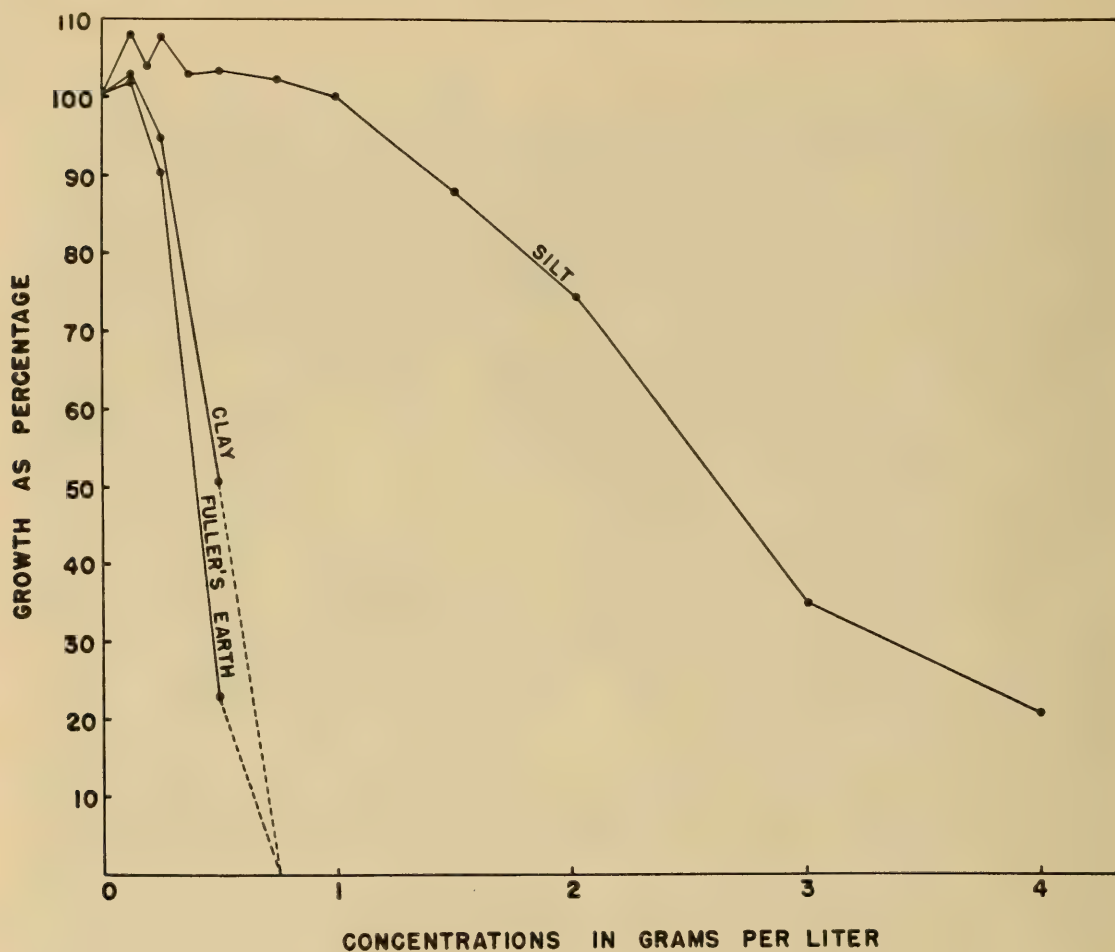


FIGURE 3. The 12-day increase in mean length of clam larvae, grown in different concentrations of suspended materials, plotted as percentages of the increase in mean length of larvae in control cultures. Each point represents the average for 50 larvae from each of duplicate cultures in each of two experiments.

dence that the larvae were taking the flagellates provided as food, and there was no growth of the larvae in a concentration of chalk as low as 0.250 g./l. With either clay or Fuller's earth, 0.500 g./l. was the highest concentration in which the larvae showed evidence of taking food (color in digestive gland) or any increase in size (Fig. 3). In cultures receiving higher concentrations of clay or Fuller's earth, many larvae ingested some of the suspended particles, apparently in sufficient quantity to block the digestive tract and cause death. Kaolin in a concentration of 0.500 g./l. caused approximately 50 per cent mortality in 12

days and almost complete mortality at all higher concentrations. There was no appreciable mortality of larvae in 0.500 g./l. of Fuller's earth in 12 days, but at all higher concentrations mortality exceeded 90 per cent.

When silt was used as the suspended material the results were quite different from those with clay, Fuller's earth or chalk. Clam larvae showed evidence of taking food (color in digestive gland) even at a concentration of 4.0 g./l. of silt, but growth was negligible, and it is doubtful if any larvae in natural waters could reach metamorphosis in either 3.0 or 4.0 g./l. of silt. Growth of most larvae was also considerably retarded by concentrations of 2.0 g./l. or even 1.5 g./l. of silt, but some larvae under these conditions had reached setting size (maximum length 185 μ and 200 μ , respectively) by the twelfth day. Growth of clam larvae was approximately normal in 0.750 g./l. of silt and, at all lower concentrations, was somewhat better than that of larvae in the control cultures (Fig. 3). There was no appreciable mortality of clam larvae, within 12 days, in any of the concentrations of silt tested.

Soil particles ranging in size from 62 microns to 4 microns are listed as silts, while particles ranging in size from 4 microns to 0.24 microns are listed as clays. From our experiments it would appear that it is the larger particles (coarse silt 62 to 31 microns) in the silt and unground Fuller's earth, or aggregates of particles in other substances that interfered with development of clam eggs, while the smaller particles, characteristic of the clays, probably had little effect on egg development. The results of experiments on growth of larvae, however, seem to indicate that the larger particles, characteristic of coarse silts, may have little effect on growth. It seems to be the smaller particles, as in clays, precipitated chalk, and finely ground Fuller's earth, which are about the size of food cells, that interfere most with growth of larvae. This interference appears to be primarily mechanical through blockage of the digestive tract, but there is some indication that it may also have been, at least in part, an indirect result of an adverse effect of the suspended materials on the food organisms.

The more rapid growth of clam larvae noted in lower concentrations of silt and at the lowest concentrations of clay and Fuller's earth is perhaps due, in part, to chelation of toxic substances, and with silt, in part, to positive growth factors. An improvement in the rate of growth of algal cultures upon addition of soil extract has long been known to botanists, and soil extract has been a basic ingredient in many of the media for algal cultures.

The author wishes to express his appreciation of the suggestions and constructive criticism given by Dr. V. L. Loosanoff, Director of Milford Laboratory. Thanks are also due the mechanical staff who constructed the wheel and constant temperature box, to Kenneth Spencer for preparing the figures and to Miss Rita Riccio for her careful editing of the manuscript.

SUMMARY

1. Some clam eggs developed normally in concentrations of 4.0 g./l. of clay, precipitated chalk or finely ground Fuller's earth, although the percentage developing normally decreased as the concentration of these suspended materials increased.

2. In silt concentrations below 0.75 g./l. the percentage of clam eggs developing normally was not significantly different from that in control cultures but decreased progressively in successively higher concentrations.

3. None of the clam eggs developed normally in silt concentrations of 3.0 or 4.0 g./l.

4. Larvae resulting from clam eggs developing in high concentrations of each of the suspended materials were reared to metamorphosis after being returned to normal sea water at 48 hours.

5. Clam larvae were unable to grow in concentrations of clay, chalk or Fuller's earth as high as those at which some eggs developed.

6. The highest concentration of chalk was 0.25 g./l. and 0.5 g./l. was the highest concentration of clay and Fuller's earth at which clam larvae showed any growth and mortality exceeded 90 per cent at all higher concentrations.

7. In a silt concentration of 0.75 g./l. growth of clam larvae was approximately normal and at lower concentrations was slightly faster than that of larvae in control cultures.

8. In silt concentrations of 1.0 to 2.0 g./l. growth of clam larvae was retarded and at 3.0 and 4.0 g./l. growth was negligible.

9. Even at a silt concentration of 4.0 g./l. there was no appreciable mortality of clam larvae within 12 days.

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CATION REGULATION AND SURVIVAL OF THE RED ALGA, PORPHYRA PERFORATA, IN DILUTED AND CONCENTRATED SEA WATER ¹

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Scott and Hayward (1953, 1954), Biebl (1956), and one of us (Eppley, 1958a, 1958b) have considered that a significant feature of ion transport in algae may lie in maintaining a relatively constant intracellular ionic environment, *i.e.*, cellular homeostasis. That transport mechanisms are of survival value is attested to by the great salinity tolerances established for intertidal algae (Biebl, 1952, 1953, 1956, 1958). Although there is increasing interest in homeostatic mechanisms in marine organisms (*e.g.*, Bullock, 1958), data on ion regulation in algae during osmotic stress are scant.

Blinks (1951) has reported the accumulation of K by *Valonia* from slowly concentrating sea water. *Ulva lactuca* loses some K in 70 per cent sea water which is reaccumulated when external Na is returned to normal (Scott and Hayward, 1955).

Ion transport has been implicated in a number of algae in addition to those mentioned. Bergquist (1958a) has studied Na and K movements after desiccation in *Homosira banksii*, MacRobbie and Dainty measured K and Na fluxes in *Rhodomenia palmata* (MacRobbie and Dainty, 1958a) and in the brackish water *Nitellopsis* (MacRobbie and Dainty, 1958b). In each case a high cytoplasmic K content and an Na content somewhat lower than that of the medium are to be noted.

To a degree, salinity tolerances of algae correlate with their intertidal zonation (Biebl, 1952). However, differences are less marked among intertidal and deeper water forms. Obviously other factors are also involved in intertidal zonation and Doty (1946) contends that the duration of exposure, *i.e.*, submergence or emergence, is of prime importance.

Doty and Archer (1950) further suggest that bright sun, rain, and freezing weather are, in that order, the most critical factors in intertidal algal survival. Kanwisher (1957) has recently reported the effects of freezing and drying on the respiration of some intertidal algae. The present paper reports a study of cellular cation concentrations in *Porphyra perforata*, a red intertidal alga, in different sea water concentrations and the role of ion transport in response to osmotic stress is discussed.

METHODS

Tissue volumes. Tissue water was taken as the difference between fresh weight (after blotting twice with tissue paper) and dry weight (24 hours at

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105° C.), divided by the fresh weight. Apparent free space (AFS) (Briggs and Robertson, 1957) was obtained by allowing $S^{35}O_4$ to diffuse into the algal tissues from sea water for two minutes, blotting twice, then placing the tissues in 10 ml. distilled water for 10 minutes. Aliquots of the distilled water containing the labelled sulfate were then counted with gas flow apparatus. The results are expressed as volume per unit fresh weight. Apparent osmotic volume (AOV) (Briggs and Robertson, 1957), corresponding roughly to the microscopically estimated cytoplasmic volume, was taken as the difference between tissue water and AFS for each sea water concentration.

Ion contents. Sodium and potassium contents were determined by flame photometry. Results are expressed in units milli-equivalents per kilogram fresh weight (meq./Kg. FW), or milli-equivalents per liter AOV.

Artificial sea waters. One hundred per cent sea water was prepared according to the following formula: NaCl, 0.55 M; $MgSO_4$, 0.027 M; $MgCl_2$, 0.027 M; $CaCl_2$, 0.01 M; KCl, 0.01 M. The concentrations were adjusted proportionately for diluted and concentrated sea waters. In some cases diluted sea water was prepared by adding distilled water to natural sea water. Calcium chloride was omitted for Ca-free sea water.

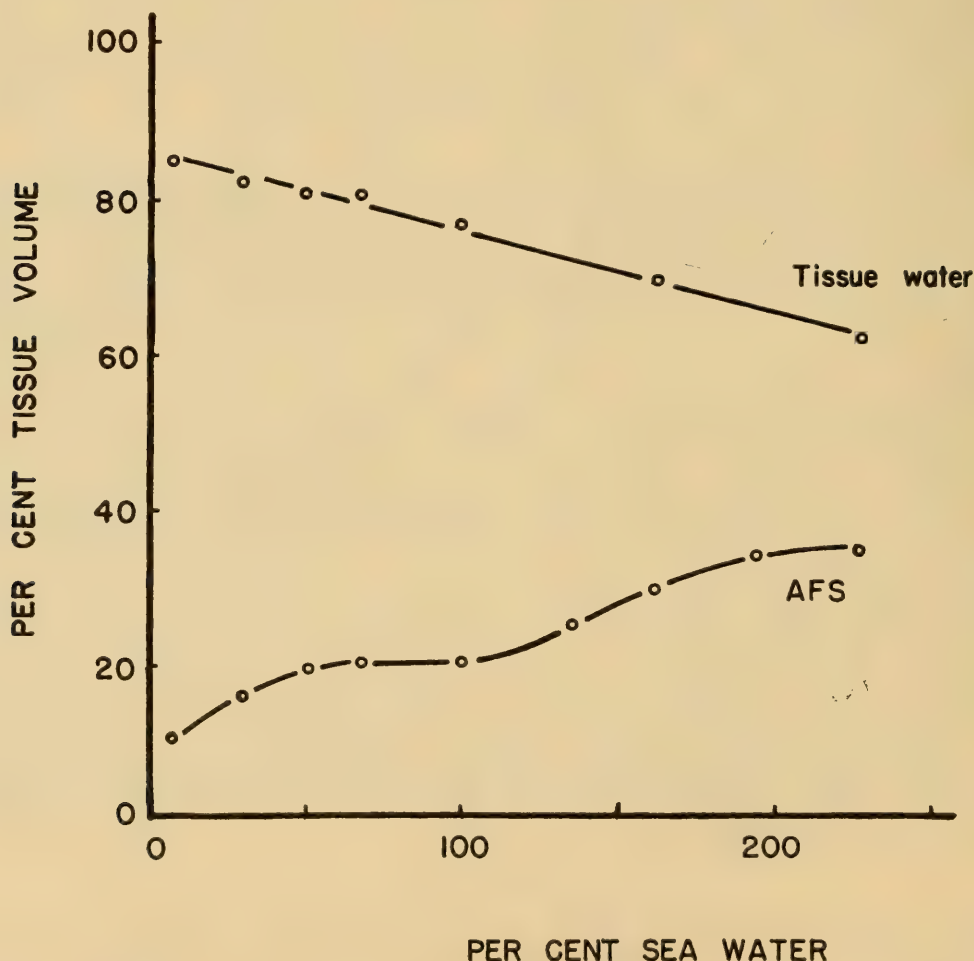


FIGURE 1. Tissue water and apparent free space (AFS) of *Porphyra perforata* as functions of sea water concentration. Apparent osmotic volume is taken as the difference between the two curves.

RESULTS

Tissue volumes at different sea water concentrations. Tissue water and AFS are shown as functions of sea water concentration in Figure 1. AOV is taken as the difference between the two curves. Individual points refer to samples taken

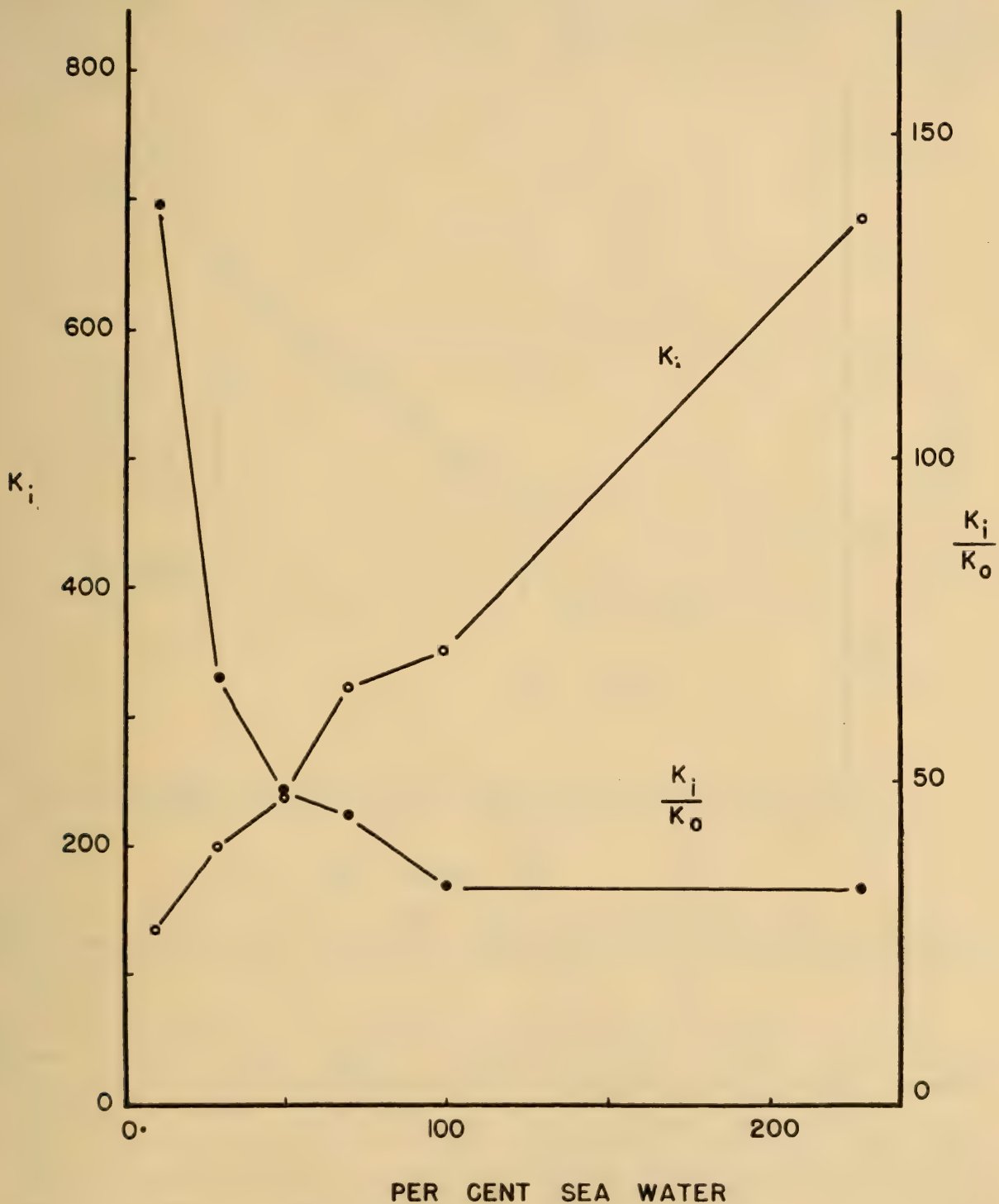


FIGURE 2. Potassium content (K_i), expressed on the basis of apparent osmotic volume (meq./L. AOV), and potassium accumulation ratios (K_i/K_o) for *Porphyra perforata* after 24 hours in different sea water concentrations.

after 24-hour exposure. However, samples taken at two minutes give identical results. Volume equilibration takes place very rapidly in the monostromatic algal tissues.

Tissue water follows a linear relationship with concentration. Dry weight provides an increasing component of tissue weight with increasing concentration. Values for weight changes at 50 and 150 per cent sea water are similar to those reported for shrinking and swelling of *Porphyra tenera* blades (Ogata and Takada, 1955).

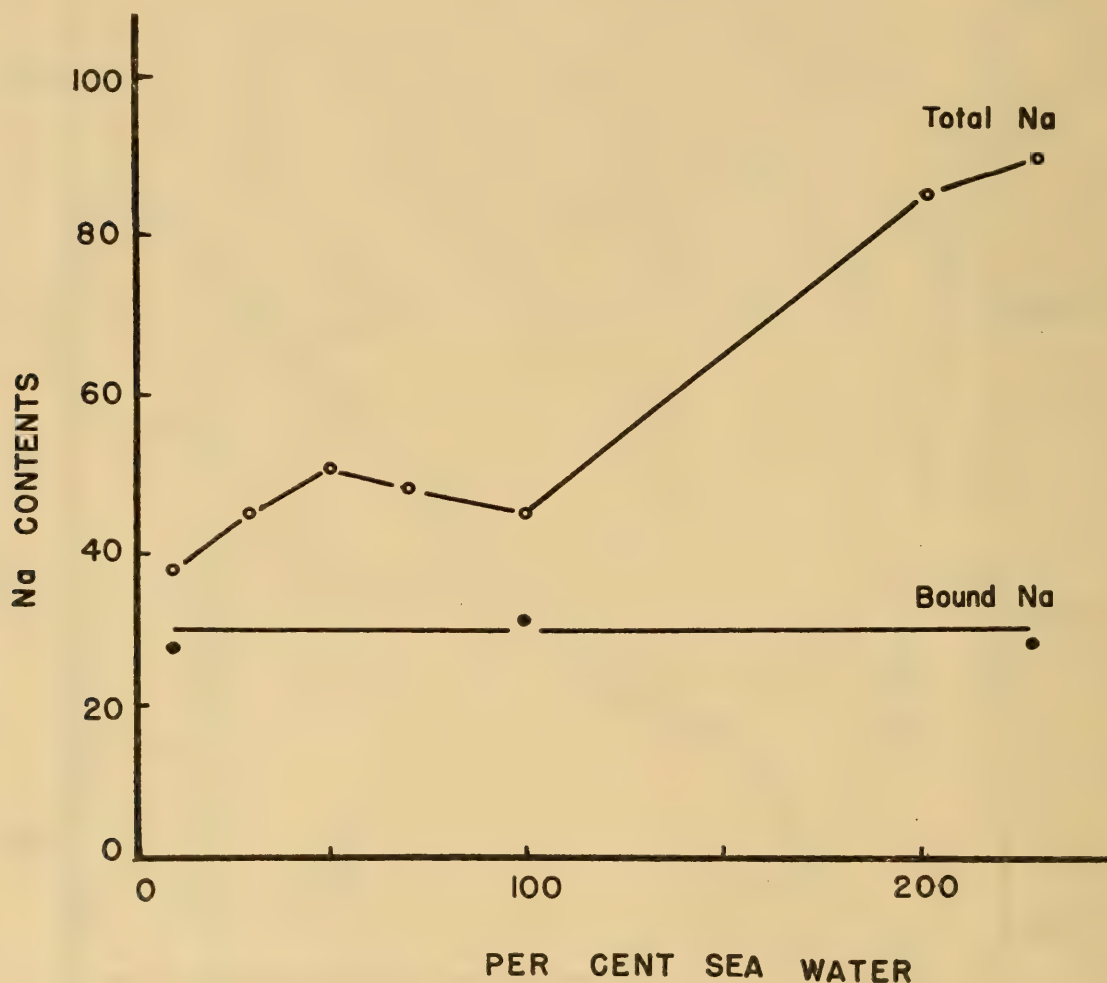


FIGURE 3. Total tissue sodium and bound sodium (the latter determined for killed tissues) in *Porphyra perforata* after 24 hours at different sea water concentrations. Units meq./Kg. FW.

Decrease in AFS in diluted sea water suggests that swelling of the cells squeezes water from the intercellular spaces. At high concentrations the reverse may hold, the extracellular material shrinking disproportionately from the cells. That the intercellular material, a galactan sulfate (Eppley, 1957), is not free to swell indefinitely is indicated by Miwa's study of *Porphyra tenera* (Miwa, 1940). In this species, and probably also in *P. perforata*, at least three structural carbohydrates exist. "Outer membranes," forming a sandwich about the blades, probably restrict swelling. Miwa found these polysaccharide membranes to be hydrolyzed only with difficulty. The galactan sulfate lies between and immediately

surrounds the cells, each of which has its own discrete wall. None of the three polysaccharides could be identified by Miwa as cellulose.

AOV, taken as an estimate of cytoplasmic volume, shows a relationship to concentration which is reverse to that followed by AFS. Swelling is noted in diluted sea water, a fairly constant volume is maintained between 50 to 100 per cent sea water, and above this value the volume decreases. The cells do not behave as perfect osmometers, nor do they show typical plasmolysis. Both these characteristics are likely due to the extracellular polysaccharides, each of which seems to have distinct swelling and shrinking characteristics. We have no evidence

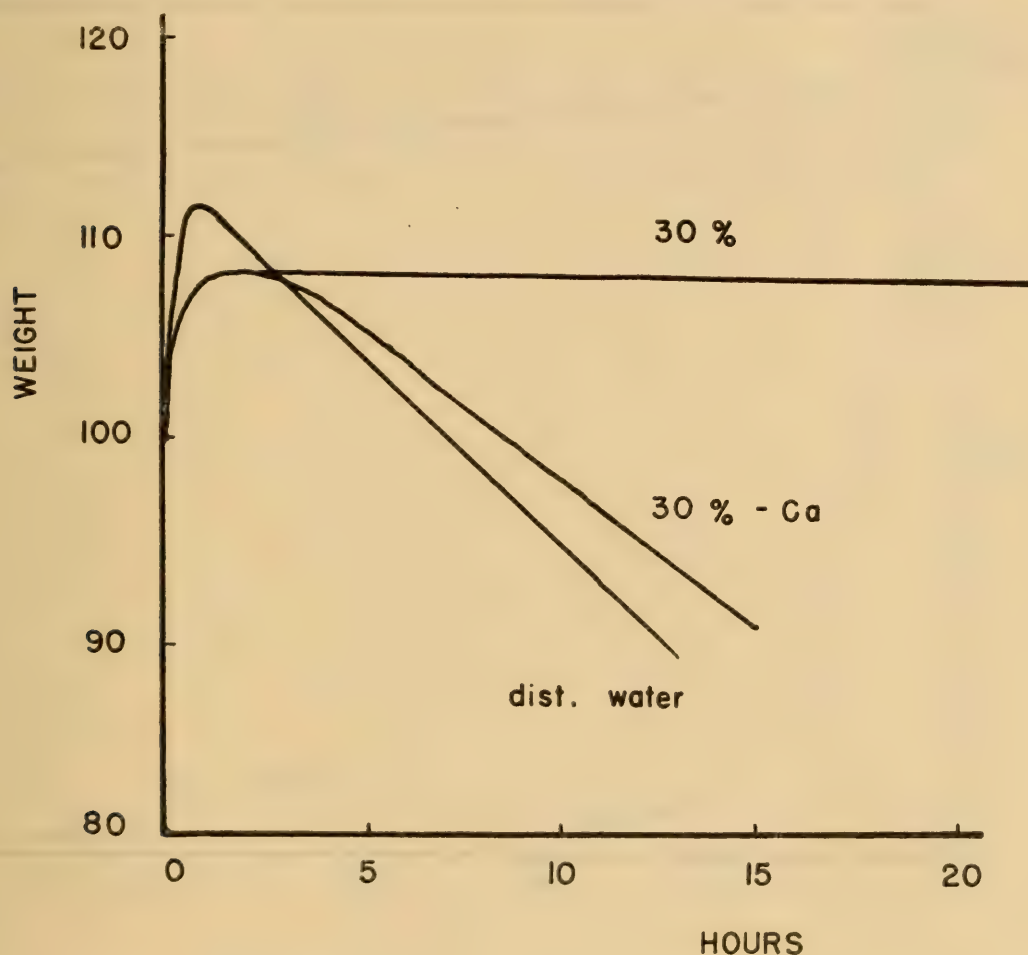


FIGURE 4. Weight, expressed as per cent of the initial weight, of *Porphyra perforata* tissues in 30 per cent sea water with Ca, 30 per cent sea without Ca, and in distilled water. Time course.

that the volume control between 50 and 100 per cent sea water is due to water secretion. Nor does there seem to be any necessity for proposing this as an explanation.

Ion contents in different sea water concentrations. K contents, expressed on an AOV basis, and K_i/K_o accumulation ratios are shown in Figure 2. Cell K follows a linear function of sea water concentration, although a 35-fold, or greater, accumulation is evident throughout. Considerable retention of K occurs in diluted sea waters, resulting in high accumulation ratios, although the actual K content

is lower here than in 100–200 per cent sea water. Little K is bound by the extracellular polysaccharides (1–2 meq./Kg. FW), although large amounts of K may be adsorbed to structural components in other algae, such as *Homosira* (Bergquist, 1958a). Potassium is the principal cellular cation of *Porphyra* at all concentrations studied.

The distribution of Na is less clear, due to appreciable extracellular adsorption. Amounts bound by killed tissues are independent of concentration of sea water for

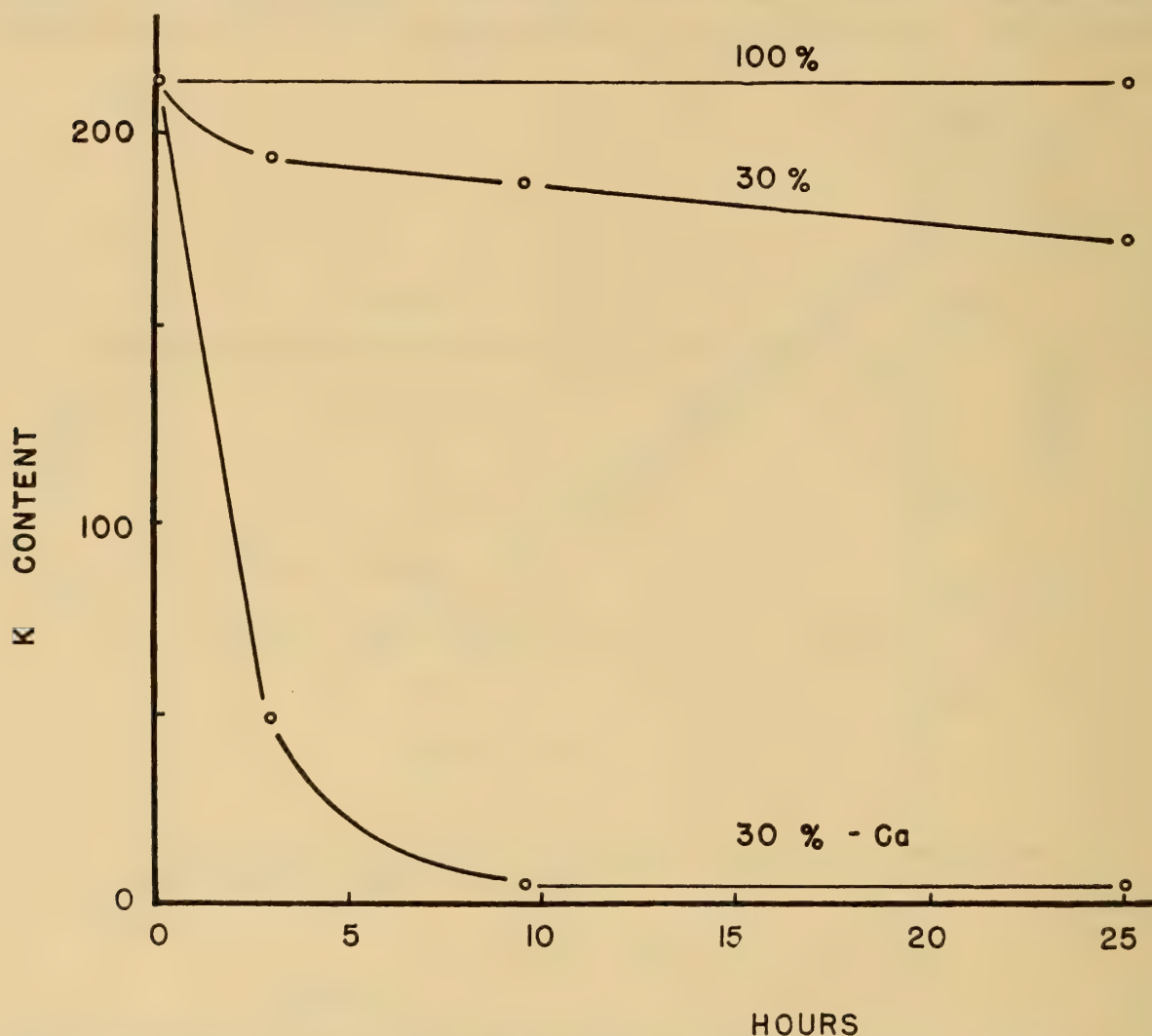


FIGURE 5. Potassium contents (meq./Kg. FW) of *Porphyra perforata* tissues in 100 per cent sea water, 30 per cent sea water with Ca, and 30 per cent sea water without Ca. Time course.

a given plant (Fig. 3), but considerable variation in bound Na is evident in different plants. Therefore Na contents are not expressed on an AOV basis. Throughout the concentration range studied, however, Na is excluded by the cells. The degree of exclusion varies with concentration, but is approximately 10-fold at 100 per cent sea water and increases with increasing sea water concentration.

Total K plus Na thus varies in a roughly linear fashion with salinity, while K is accumulated and Na partially excluded throughout. A similar situation is re-

ported for several halophilic bacteria (Christian and Ingram, 1959). In these bacteria the freezing point depression of cell sap varies directly with sea water concentration, with K contributing significantly to cell osmotic pressure, as does Na.

Calcium and survival in diluted sea water. Tissues placed in 30 per cent sea water without calcium, or in distilled water (Fig. 4) lose weight rapidly after an initial increase. Addition of calcium chloride, 0.01 *M*, prevents the weight loss.

Calcium-free 30 per cent sea water also results in rapid loss of K (Fig. 5), while with Ca the K loss is much depressed. Cell Na, data not shown, increases slightly without Ca (with the concentration gradient), but remains at a lower and constant value with Ca.

TABLE I

Potassium accumulation and sodium extrusion in the presence and absence of CaCl₂ or SrCl₂ (10 mM/L.). Tissues were first agitated in K-free sea water 20 to 30 hours to render them low in K and high in Na so that subsequent net transport could be measured. They were then transferred to sea water plus KCl.

Expt.	Duration of expt. in hours	K uptake meq./Kg. FW	Na extrusion meq./Kg. FW
1	1.5		
initial		0	0
-Ca		59	26
+Ca		55	38
2	1.5		
initial		0	0
-Ca		46	41
+Ca		60	57
3	19.5		
initial		0	0
-Ca		-7	13
+Ca		96	47
4	16.5		
initial		0	0
-Ca		15	13
+Ca		50	31
+Sr		57	33

Calcium in net transport of K and Na. To investigate the role of Ca in net uptake of K and extrusion of Na, tissues were rendered low in K and high in Na by soaking them in K-free sea water (Eppley, 1958b). They were then placed in sea water with KCl (10 or 20 meq./L.) and the ion contents of the tissues were followed in time. Initial uptake of K and extrusion of Na (Table I) is independent of Ca presence. However, after longer periods net active movements are reduced, indicating loss of K and gain of Na in the absence of Ca. Strontium (SrCl₂, 0.01 *M*) may substitute for Ca in preventing loss of K and gain of Na.

Our results are best explained by assuming that Ca-lack gradually brings about leakage through the cell membranes, resulting in increasing movements of salts along their concentration gradients. It seems unlikely that Ca plays any direct

role in the operation of ion transport mechanism, *i.e.*, active transport, but it could well be required to maintain K:Na selective sites on the membrane, or participate in labile membrane structure.

DISCUSSION

Tissue volumes. Because of the lack of a central vacuole and because microscopic estimates of AFS correspond roughly to those measured with $S^{35}O_4$, we have assumed that AFS in *Porphyra* includes the extracellular water but no cytoplasmic component, as is suggested for some higher plant tissues (Briggs and Robertson, 1957). With these assumptions any tissue water not corresponding to AFS must be cellular. AFS has been measured with sucrose for tissues in 100 per cent and diluted sea waters, with results identical to those obtained with radiosulfate. Whether the experimental values include all the extracellular water may be open to question, as well as whether a cytoplasmic component is included. Interestingly, Bergquist (1958b) reported that KCN increases AFS values in *Homosira*. Whether this represents an actual increase in AFS or modification of anionic binding sites was left an open question.

It is with the above reservations that we have taken AOV as the difference between tissue water and AFS, and have calculated K concentrations on this basis.

We do not necessarily presume a uniform cytoplasmic distribution of K, although we plan to investigate this point further. Some localization may occur in vacuomes which are variable in number, but which may comprise a small fraction of cytoplasmic volume. These are visible with neutral red staining. Mitochondria, the chromatophores, and local membrane vesiculations, if they occur, could be sites of variation in cytoplasmic ion concentrations.

While *Porphyra* cells do not behave as perfect osmometers, our results are consistent with the view that active water movements do not take place. Variations in AOV with salinity seem to be due to different degrees of shrinking and swelling of the protoplast and the extracellular, structural polysaccharides.

Ion regulation in concentrated sea water. In corroboration of Biebl's findings (Biebl, 1953), we find that *Porphyra* may survive for some time in 200 per cent sea water. Over the studied range, external ion concentrations of the medium increase equally. Selective precipitation of salts, *i.e.*, $CaSO_4$, occurs only with concentration above 300 per cent. Our investigations have not extended to such concentrations.

Accumulation ratios for K are identical for 100 and 200 per cent sea water. Cell Na shows some increase with concentration, but is lower than that of the medium; thus cation selectivity is retained.

In these experiments changes in salinity were abrupt. Coenocytic algae such as *Valonia* (Blinks, 1951) do not tolerate such rapid changes. Lack of a large central vacuole in *Porphyra* cells may be of importance in this regard. There is no plasmolysis and thus no mechanical injury to the cell membranes with rapid salinity change. AFS and AOV adjustments are almost immediate, as indicated by identical AFS values at two minutes and 24 hours of exposure to concentrated sea water.

Porphyra perforata grows between the 3- and 3.5-foot tide levels, referred to San Francisco (Doty, 1946). Here that alga is regularly covered and uncovered

by the tide twice daily. The maximum period of exposure is 6–8 hours. Concentration of sea water between the algal blades during this period seems unlikely to exceed that tolerated in our experiments, even on the hottest days of summer. Thus we feel that osmotic stress under these conditions is insufficient to result in breakdown of ion transport, resulting in death. Survival in concentrated sea water encountered in the field is not a problem, in our view.

A more serious problem to the emerged algae on hot days is heating, and possibly photooxidation. In summer the superficial algal blades are bleached. Underlying blades (the blades overlap one another) may survive.

Transport and survival in diluted sea water. A 6–8-hour exposure to rain could result in serious injury or death, as indicated by our experiments. Lack of Ca results in rapid loss of K and of weight, and these losses are speeded during osmotic stress. In 100 per cent sea water, free of Ca, *Porphyra* may survive up to 30 hours, compared with only 6–8 hours in 30 per cent Ca-free sea water.

Lack of potassium (Eppley, 1958b) also may influence survival because of the necessity of K for extrusion of Na. Unpublished results indicate that respiration is inhibited about 60 per cent in the absence of Na. Thus the presence of Ca, K, and Na (and probably also Mg) is required for normal operation of cellular processes, one of which is ion transport.

It is of some interest that even in distilled water *Porphyra* tissues adsorb about 10 mM/Kg. FW of Ca. This amount, probably associated with the extracellular galactan sulfate, is not apparently available to the critical sites involved in maintaining membrane selectivity. The implication of this finding may be of some importance in clarifying the role of trace elements passively adsorbed to extracellular polysaccharides in algae. Thus trace element "accumulation" (Black and Mitchell, 1952), in which the extent of adsorption was not determined, might be re-interpreted in the light of this result, with respect to the survival value of such "accumulation." The likelihood remains, however, that in the presence of exchangeable cations, adsorbed trace elements could be made available to the cell surfaces for real accumulation.

Growth experiments which might clarify this problem have not been possible, due to our failure to obtain growth of *Porphyra* in the laboratory. The recent results of Kanazawa and Kashiwada (1959) on the culture of *Porphyra tenera* cast some hope on our aspirations of doing so, however.

A heavy rain, by washing away the sea water film normally present between and around the blades even during emergence, would almost certainly abolish membrane selectivity, decrease respiration, induce loss of cellular cations, and result in high mortality if the blades were exposed long enough. In southern California *Porphyra perforata* largely disappears in the winter, and rain may be involved in this mortality. Other factors are also involved, however.

At the site studied, approximately two miles north of the Los Angeles-Ventura County boundary in California, mass movements of sand were observed during the winter of 1958–1959. The old *Porphyra* bed was entirely covered and a new bed appeared about 50 yards south in the following spring. A smaller sand movement partially covered the new bed in July, 1959. While the extracellular polysaccharides may act as cushions against moderate wave impact and sand scouring, the plants may be torn loose or shredded in a heavy surf or be buried by sand

movements. Such physical processes may be important in survival and distribution as well as physiological tolerances.

SUMMARY

1. Over a wide range of salinity (10 to 200 per cent sea water) cells of *Porphyra perforata* accumulate K and partially exclude Na. Apparent osmotic volume is nearly constant between 50 and 100 per cent sea water. This imperfect volume control is thought to be due to differential shrinking and swelling of structural polysaccharides and not to active water secretion.

2. Survival of *Porphyra* in diluted and concentrated sea water is discussed with respect to ion transport. Rain is potentially a more serious threat to survival, due in part to breakdown of ion transport, than is concentration of sea water. Calcium seems especially important in maintaining membrane selectivity toward K and Na.

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THE EFFECTS OF THYROXIN AND GROWTH HORMONE ON LIVER POLYPLOIDY ¹

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Polyploidy in the cells of the hepatic parenchyma is known to be influenced by changes in the endocrine state produced by hypophysectomy and growth hormone administration (Di Stefano and Diermeier, 1956; Leuchtenberger, Helweg-Larsen and Murmanis, 1954). We have confirmed the effects of both the former (Geschwind, Alfert and Schooley, 1958) and the latter (see below) on liver polyploidy, but have shown that although the hormonal environment may normally play a role in the development of polyploidy, it is not indispensable to that development (Geschwind, Alfert and Schooley, 1958).

In the course of experiments designed to investigate the hormonal factors necessary to stimulate growth in the genetic dwarf mouse (Cole, Geschwind, and Bern, unpublished experiments), it was found that highly purified bovine growth hormone preparations were far less effective in stimulating body growth than were the crude preparations used for this purpose by Fønss-Bech (1947). Since contamination of the crude preparations with the thyrotropic hormone was strongly suspected, it was decided to investigate the effects of thyroxine (more readily available in pure form than is the thyrotropic hormone), alone and in combination with growth hormone, on body growth and liver polyploidy. The results of the experiments on liver polyploidy in these animals, together with the results of a similar series of experiments in the hypophysectomized rat, are reported below.

MATERIALS AND METHODS

Male and female dwarf mice, 40 to 65 days of age, were apportioned into four groups, one of which served as a control group. The animals in each of the other groups were injected with either 50 micrograms twice weekly of dl-thyroxine (the dose suggested by Nielsen, 1953), 25 micrograms daily of a bovine growth hormone preparation or both hormones at the aforementioned dose levels. Injections were continued for 21 days.³

Male rats of the Long-Evans strain were hypophysectomized at 28 days of age. One week later the animals were apportioned into four groups, one of which served as a control group. The animals in each of the other groups were injected with either 2 micrograms daily of l-thyroxine, 25 micrograms daily of the bovine growth hormone preparation, or both hormones at these respective dose levels. Injections were continued for 22 days.

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All animals were sacrificed on the day after the last injection, and the hepatic left lateral lobes were removed from the anesthetized animals, sliced freehand, and fixed in acetic-alcohol. The procedure followed for the preparation of sections for counting and the actual counting procedures have been previously described (Alfert and Geschwind, 1958). Approximately 1000 parenchymal cells were scored in the sections obtained from each animal. The mean percentage distributions of the various types of hepatic parenchymal cells in each of the experimental groups in both series, along with the mean initial and terminal body weights of the animals in each group are recorded in Tables I and II.

RESULTS AND DISCUSSION

In the genetic dwarf mouse, the administration of either growth hormone or thyroxine results in a decrease in the number of hepatic mononucleate diploid cells and an increase in tetraploid and binucleate cells (Table I). Of the two hormones, thyroxine, at the dose administered, appears to be more effective in producing these changes. By far the greatest effect, however, was observed in those animals receiving the combined hormone treatment. Such animals also showed the greatest weight gain during the period of injections.

It was difficult to determine unequivocally the manner by which thyroxine brought about its observed effect, since at least three different interpretations of the data could be advanced. One is that thyroxine enhanced polyploidization directly, either by acting as a specific mitogenic agent or by promoting the need for accelerated liver function. A second interpretation might be that the changes in the liver were simply the result of the administration of a large dose of a compound that had to be conjugated and metabolized by the liver, or that was toxic

TABLE I

The distribution of hepatic parenchymal cell types in control and experimental dwarf mice

Group	Body weight		Mean percentage distribution of cell types*				
	Initial	Terminal	2n	2n bi	4n	4n bi	8n
Control**	g. 6.1	g. 7.1	81.4 (78.2-83.1)†	13.1 (10.5-15.3)	3.9 (1.7- 5.2)	0.1 (0.0-0.2)	0.1 (0.0-0.3)
Thyroxine	6.5	10.0	53.4 (49.0-60.4)	18.1 (12.2-23.7)	24.3 (21.9-26.1)	3.0 (1.4-4.8)	0.1 (0.0-0.2)
Growth hormone	7.1	10.3	69.0 (65.4-70.9)	20.9 (17.0-23.2)	8.1 (4.1-10.3)	0.9 (0.6-1.1)	0.0
Thyroxine + growth hormone	6.2	12.1	31.6 (23.4-37.9)	8.6 (5.6-10.2)	52.8 (45.7-61.7)	4.2 (3.3-5.7)	1.6 (1.0-2.3)

* 2n = diploid; 2n bi = diploid binucleate; 4n = tetraploid; 4n bi = tetraploid binucleate; 8n = octaploid. (In all experiments approximately 1% of the cells could not be classified.)

** Three animals per group.

† Range of individual results.

TABLE II

The distribution of hepatic parenchymal cell types in control and experimental hypophysectomized rats

Group	Body weight		Mean percentage distribution of cell types*				
	Initial	Terminal	2n	2n bi	4n	4n bi	8n
Control**	g 74	g 82	70.1 (67.3-74.5)†	24.0 (20.6-26.5)	4.8 (4.1-5.8)	0.2 (0.1-0.4)	0.0
Thyroxin	77	93	52.3 (46.6-57.8)	24.0 (18.1-26.9)	22.0 (19.4-24.6)	0.7 (0.1-1.1)	0.1 (0.0-0.2)
Growth hormone	74	103	53.2 (50.7-58.1)	24.0 (19.9-29.6)	20.6 (17.3-23.6)	0.8 (0.1-1.2)	0.1 (0.0-0.4)
Thyroxin + growth hormone	72	123	22.1 (19.8-26.3)	9.5 (6.4-14.7)	61.4 (56.0-68.1)	3.9 (2.4-6.2)	1.9 (1.6-2.1)

*, **, † Symbols have the same meaning as in Table I.

to the parenchymal cells in the dosage employed. Finally, it was possible that thyroxin, in its capacity as a hormone, promoted repair of pituitary acidophiles which are normally absent in the dwarf mouse (Ortman, 1956), and by so doing stimulated growth hormone secretion. There is good evidence that thyroxin repairs pituitary acidophile cytology in the thyroidectomized rat (Koneff, Scow, Simpson, Li and Evans, 1949; Contopoulos, Simpson and Koneff, 1958), and concomitantly restores growth activity (Eartley and Leblond, 1954; Contopoulos *et al.*, 1958). Such an effect on the acidophiles could explain the findings of Carrière (1955) that in the thyroidectomized rat, hepatic nuclei with diameters of only 6.0 μ are found after a considerable post-operative interval, whereas in normal controls or in thyroidectomized animals treated with growth hormone, thyroxin, or both, nuclei with diameters of 6.5 and 8.0 μ are seen. The probability that growth hormone secretion is depressed after thyroidectomy could also explain our own unpublished findings that in animals which had been thyroidectomized at 24 days of age, the distribution of hepatic parenchymal cell types found at 114 days was that characteristic of 40- to 45-day-old animals. The change, in the normal animal, in the distribution of cell types with age has been previously reported (Alfert and Geschwind, 1958).

In order to eliminate the possibility that thyroxin was acting to enhance polyploidization by repairing pituitary function, experiments were conducted in hypophysectomized rats. Such animals have a further advantage of being far more sensitive to thyroxin than is the dwarf mouse, permitting a much smaller dose of thyroxin to be employed (2 micrograms daily to a rat weighing 75 grams versus 50 micrograms twice weekly to a mouse weighing 6 grams). Thus, any effects due to conjugation and metabolism by the liver, or to toxicity, are minimized.

The results of these experiments (Table II) reveal that the effect of thyroxin on the hypophysectomized rat is comparable, at the dose levels chosen, to that

obtained with growth hormone. In both cases a 25% decrease in the number of mononucleate diploid cells has occurred, along with a 5-fold increase in the number of tetraploid cells. As in the case of the dwarf mouse, the greatest effect, however, was found in those animals receiving both hormones, for in the livers of such animals the total number of diploid cells was only one-third that found in the control animals, while the numbers of both tetraploid and octaploid cells increased markedly. The synergistic effect of the two hormones administered together is also reflected in the body weight increments of the treated animals.

These experiments therefore indicate that thyroxine (and presumably thyrotropic hormone) as well as growth hormone contributes to the hormonal environment which affects the development of hepatic polyploidy. It should also be noted that thyroxine alone can be at least as efficient as growth hormone in promoting the progression of hepatic polyploidy; thus, as we also have demonstrated by the results of a previous experiment (Geschwind, Alfert and Schooley, 1958), the suggestion of Leuchtenberger *et al.* (1954), concerning the supposed essential role of growth hormone in somatic polyploidy, cannot be maintained.

SUMMARY

1. The effect of thyroxine and of growth hormone on liver polyploidy has been investigated in the dwarf mouse and in the hypophysectomized rat.

2. Either hormone, acting alone, stimulated polyploidization in these experimental animals; the combination of both hormones synergized to produce an hepatic cell picture dominated by higher polyploid classes.

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SURFACE ANTIGEN DYNAMICS IN THE SLIME MOLD, *Dictyostelium discoideum*¹

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The mechanisms by which cells in a morphogenetic system effect coordinated movements and specific associations constitute basic developmental problems that have been the subject of many investigations. That such mechanisms possibly involve forces similar to those existing between antigens and antibodies was recognized and elaborated by Tyler (1947, 1957), Weiss (1947), and Spiegel (1954a, 1954b). A recent comprehensive review of the problems involved in such morphogenetic processes as cell adhesion and cell migration has been presented by De Haan (1958) and need not be recapitulated here.

Morphogenetic expression in the slime mold, *Dictyostelium discoideum*, requires oriented movements of cells, a process which involves changes in shapes of the cells. The stimulus for the changes in shape may be an external one (via acrasin) in which large numbers of cells are affected from a common direction simultaneously (Bonner, 1947, 1950). Following the aggregation process the cells are organized into a pseudoplasmodium in which the cell surfaces are in intimate association with each other. Throughout morphogenetic movements the cells maintain contact with each other. Acquisition of the ability to adhere to one another implies a change in the nature of the surface of the cells. Consistent with this is the evidence obtained by Gregg (1956) that new antigens appear at the onset of aggregation.

It is the purpose of this study to investigate changes in the surface antigens of the cells from the beginning aggregation stage to the formation of the mature spores. Such a study might be expected to reveal not only the pattern of antigens during morphogenesis but something of the possible role of surface antigens in maintenance of the integrity of the pseudoplasmodium and in regulation of the course of cellular movement.

METHODS AND MATERIALS

The slime molds were cultured according to the general methods employed by Bonner (1947) with the exception that the bacterial associate was *Aerobacter aerogenes* rather than *Escherichia coli*. The cultures were maintained at 22° C. during the growth phase. During the period of aggregation they were transferred to a 17° C. incubator, the lower temperature of which extended the migration and culmination phases.

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Preparation of injection-antigens

Migrating pseudoplasmodia were collected by cutting discs of nutrient agar bearing aggregates on the verge of migration. These discs were transferred to similar sized spaces in plates on non-nutrient agar (Gregg and Bronsweig, 1956). After the migrating pseudoplasmodia had accumulated on the non-nutrient surface they were collected individually with a hair loop and transferred into 1.0% NaCl.

The mature spores were collected by sweeping the tops of the mature sorocarps with a 3-mm. glass rod. The spores adhered to the glass rod and were transferred into 1.0% NaCl.

An antigen was also prepared by washing mature spores in 0.5% NaCl for 10–15 minutes. The spores were then centrifuged and the supernatant carefully removed to serve as an antigen.

These collecting procedures enabled migrating pseudoplasmodia, mature spore, and mature spore surface antigens to be obtained essentially free of *Aerobacter*. The antigens were stored in 1.0-ml. aliquots in a deep freezer at minus 15° C.

Each of the three types of antigens contained 0.2 mg. N/ml. Two rabbits each were injected with the three types of antigens. The injections were given every other day until a total of nine injections per rabbit had been given (0.5 ml. antigen/injection).

Preparation of antisera and normal (pre-injection) sera

The rabbits were bled by heart puncture five days following the final injection. The blood was allowed to stand for approximately two hours at room temperature before the clot was ringed, then placed in the refrigerator overnight before centrifugation. Complement was destroyed by heating the antisera at 56° C. for 30 minutes. The antisera were divided into 1.0-ml. aliquots and placed in the deep freezer at minus 15° C.

The normal (pre-injection) sera were obtained and prepared in the same manner as described for the antisera.

Preparation of absorbed antisera

The amoebae or spores were harvested from the culture plates by the methods described under the sections concerning the preparation of injection-antigens and test-antigens. However, in the absorption procedure all of the supernatant was removed from the packed cells. In a typical absorption, packed cells were mixed with the particular antiserum in the ratio of one volume of cells to one volume of antiserum. The preparation was allowed to absorb at room temperature for two hours followed by 22–24 hours at 10° C. The absorbed serum was then centrifuged in an electric micro-centrifuge (Microchemical Specialties Co.) at 9500 rpm for 15 minutes. The serum was then carefully removed from the packed cells and stored at minus 15° C. until desired.

Preparation of test-antigens for agglutination

These antigens were prepared by alternately washing and centrifuging (125 *g*) aggregating amoebae or spores twice in 0.5% NaCl. The resulting sedimented

cells were diluted with about one volume of 0.5% NaCl (approximately 2.5×10^8 cells/ml.) for use in agglutination tests. The cells were placed in agglutination chambers in 10.0- μ l. volumes (Gregg and Trygstad, 1958). The antiserum or normal (pre-injection) serum was mixed with the cells in 10.0- μ l. volumes with

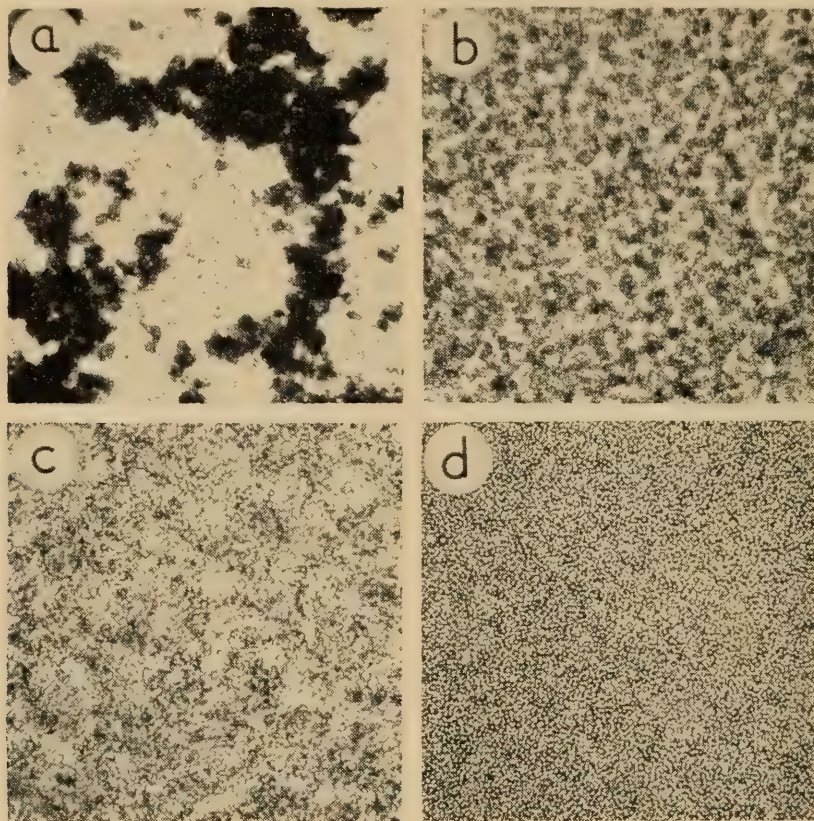


FIGURE 1a. *D. discoideum* amoebae + spore antiserum (magnification $\times 36$). 1b. *D. discoideum* amoebae + spore normal serum (magnification $\times 36$). 1c. *D. discoideum* spores + spore antiserum (magnification $\times 100$). 1d. *D. discoideum* spores + spore normal serum (magnification $\times 100$).

a small glass rod. The presence or absence of antibodies in the serum was detected by agglutination of the amoebae or spores to various degrees (Fig. 1a, b, c, d).

All antisera and normal (pre-injection) sera were tested on amoebae or spores independently. However, the results obtained from antisera produced in response

TABLE I

Antiserum titer obtained by agglutination of cells

Type of antiserum	Type of cell agglutinated	Highest dilution of antiserum
MA	Amoebae	1:80
SA	Amoebae	1:80
SSA	Amoebae	1:20

to the same type antigen or normal (pre-injection) sera have been pooled. The titers of the various sera were obtained by determining the least concentration of antiserum diluted with 1.0% NaCl, which would produce detectable agglutination of amoebae or spores (Table I).

Determination of the amounts of antigenic material extracted from the surfaces of the cells

For reasons which will become apparent it was necessary to determine quantitatively the amounts of antigenic material which may be extracted from the surfaces of the cells composing migrating pseudoplasmodia and the mature spore masses. These determinations were made in two ways as follows:

A. 1. Ten to fifty migrating pseudoplasmodia (non-nutrient agar migrants) or mature spore masses placed in 20 μ l. of 0.5% NaCl.

2. Cell surface materials extracted by gentle stirring for 10–15 minutes.

3. Cells centrifuged 5 minutes at 4600 rpm in the micro-centrifuge.

4. Washed cells and surface wash analyzed separately for total N (Bruehl *et al.*, 1946; Gregg, 1950) (Table IV).

B. 1. One hundred to five hundred migrating pseudoplasmodia or mature spore masses were placed in 100–500 μ l. 1.0% NaCl.

2. Cell surface materials extracted by gentle stirring for 10–15 minutes.

3. Cells centrifuged 15 minutes at 9500 rpm in the micro-centrifuge.

4. The surface wash was removed and saved for analysis.

5. The washed cells were analyzed for total N.

6. The volume of each surface wash from each type of cell was adjusted such that $\frac{\mu\text{l. surface wash}}{\mu\text{g. N of washed cells}} = \text{unity}$. This dilution procedure resulted in the production of surface wash in volumes proportional to the number of cells (total N) from which it was extracted.

7. The surface washes were then subjected to conventional procedures to determine optimum proportions with various antisera in precipitin tests.

8. Fifty μ l. of each surface wash and the optimum amount of each antiserum, determined in step 7, were mixed in 3 mm. $\times$ 40 mm. test tubes and allowed to incubate for 1–2 hours at room temperature. After incubation in a refrigerator overnight the precipitates were centrifuged at 9500 rpm for 15 minutes in the micro-centrifuge. The precipitates obtained were washed once with 1.0% NaCl and analyzed for total N (Table V).

RESULTS

Migrating pseudoplasmodia-antiserum (MA)

The MA effected maximal agglutination of both amoebae and spores (Table II). It should be noted here, however, that spores do not exhibit the massive type of agglutination obtained with amoebae. The maximal agglutination in spores amounts to an easily detectable regular pattern of small clumps of spores, but never the large masses of drastically clumped cells as observed when agglutinating amoebae.

Migrating pseudoplasmodia-antiserum absorbed with amoebae (MAa)

The MAa did not agglutinate amoebae or spores (Table III).

Migrating pseudoplasmodia-antiserum absorbed with spores (MAs)

The MAs did not agglutinate amoebae or spores (Table III).

Migrating pseudoplasmodia normal (pre-injection) serum (MN)

The MN used as controls in all agglutination tests along with MA, MAa, and MAs, did not effect agglutination of either amoebae or spores (Tables II, III).

Mature spore-antiserum (SA)

The SA agglutinated both amoebae and spores to the degree designated as maximum for each type of cell (Table II) (Fig. 1a, c).

TABLE II

Degree of agglutination effected by various antisera and normal (pre-injection) sera on amoebae and spores

+ = maximum agglutination; $\pm$ = minimal agglutination; and - = no agglutination. All sera were diluted to one-half of full strength as a result of the addition of amoebae. The numbers opposite the degree of agglutination values refer to the number of times a particular pattern was observed.

	Type of sera	Amoebae Degree of agglutination			Spores Degree of agglutination*		
		+	$\pm$	-	+	$\pm$	-
Antisera	Migrating	49				26	
	Spore	40				24	
	Spore surface	21				13	
Normal sera	Migrating			48			24
	Spore			39			22
	Spore surface			19			15

* In this table and Table III, $\pm$ represents the maximal degree to which spores may be agglutinated.

Mature spore-antiserum absorbed with amoebae (SAa)

The SAa failed to agglutinate amoebae, but effected maximal agglutination of spores (Table III).

Mature spore-antiserum absorbed with spores (SAs)

The SAs failed to agglutinate either amoebae or spores (Table III).

Mature spore normal (pre-injection) serum (SN)

The SN used as controls in all agglutination tests along with SA, SAa, and SAs did not effect agglutination of either amoebae or spores (Tables II, III) (Fig. 1b, d).

Mature spore surface antiserum (SSA)

The SSA effected maximal agglutination of both amoebae and spores (Table II).

TABLE III

Degree of agglutination effected by various absorbed antisera and normal (pre-injection) sera on amoebae and spores

$+$ = maximum agglutination; $\pm$ = minimal agglutination; and $-$ = no agglutination. All sera were diluted to one-half of full strength as a result of the addition of amoebae. The numbers opposite the degree of agglutination values refer to the number of times a particular pattern was observed.

	Type of sera	Amoebae Degree of agglutination			Spores Degree of agglutination*		
		+	$\pm$	-	+	$\pm$	-
Antisera abs. with amoebae	Migrating			26			10
	Spore			24		11	
	Spore surface			3		3	
Antisera abs. with spores	Migrating			9			4
	Spore			14			4
	Spore surface			7			3
Normal sera	Migrating			34			12
	Spore			38			12
	Spore surface			10			4

* See footnote to Table II.

TABLE IV

Comparison of total N in surface antigens of migrating pseudoplasmodia amoebae and mature sorocarp spores

Cell type	No. of exps.	μ g. whole cell N	μ g. surface antigen N	$\frac{\mu\text{g. surface antigen N}}{\mu\text{g. whole cell N}}$	Ratio of antigen N $\frac{\text{Spore}}{\text{Amoebae}}$
Spores	6	133.3	31.0	0.23	1.5
Amoebae	4	53.9	8.0	0.15	

TABLE V

Comparison of total N precipitated from surface antigen preparations of migrating pseudoplasmodia amoebae and mature sorocarp spores by antibodies

	No. of exp.	Surface antigens obtained from		Ratio of precipitate N $\frac{\text{Spore}}{\text{Amoebae}}$
		Amoebae	Spores	
μ g. N precipitated by spore surface anti-serum minus controls (normal serum)	3	6.8	30.0	4.4
μ g. N precipitated by migrating pseudo-plasmodia antiserum minus controls (normal serum)	3	3.0	8.0	2.7

Mature spore surface antiserum absorbed with amoebae (SSAa)

The SSAa failed to agglutinate amoebae but effected maximum agglutination of spores (Table III).

Mature spore surface antiserum absorbed with spores (SSAs)

The SSAs failed to agglutinate either amoebae or spores (Table III).

Mature spore surface normal (pre-injection) serum (SSN)

The SSN did not effect agglutination of either amoebae or spores (Tables II, III).

The amounts of surface material removed from cells composing migrating pseudoplasmodia and mature spores

It was found that in terms of total N of the surface washes, 1.5 times more material was washed from the cells composing the spore masses than from the amoebae composing migrating pseudoplasmodia (Table IV). It was assumed that the N content of the surface washes would serve as an indicator of the amount of antigenic or surface material which had been removed from the cells.

When surface washes of the two types of cells were precipitated with antisera the precipitates obtained from spore surface wash proved to be 2.7–4.4 times greater than those from amoebae surface wash (Table V). This indicates that more antigenic material was removed from the cells composing spore masses than from those composing migrating pseudoplasmodia.

DISCUSSION

Antisera produced in rabbits to antigens composed of cells and washings of cells from various stages of the slime mold, *D. discoideum*, effect agglutination of amoebae and spores. Analysis of the pattern of agglutination effected by the various types of sera on amoebae and spores demonstrated that qualitatively identical surface antigens are present during the transition from the beginning aggregate to the mature spore. However, absorption of various antisera by amoebae and spores has disclosed the presence of a surface antigen(s) in the spores which is (are) not common to early aggregates or migrating pseudoplasmodia. It is possible that the new antigen is composed of the polysaccharide with which spores become coated during the culmination process (Raper and Fennell, 1952).

An examination of Figure 1 reveals that the maximal degree to which amoebae may be agglutinated differs from that degree to which spore cells may be agglutinated. It may be seen that spores agglutinate poorly even with mature spore antiserum as compared with the agglutination of amoebae by the same antiserum.

Shaffer (1958) has demonstrated that *D. discoideum* amoebae become strongly adhesive at the onset of aggregation. Furthermore, it has been observed during the course of the present work that the amoebae composing a migrating pseudoplasmodium do not spread readily upon a glass rod. The failure to spread has been interpreted as being due to the adherence of the cells to each other. However, the cells composing a spore mass tend to spread readily when tested in the same fashion, suggesting that the adhesive properties of the spores are relatively poor.

In seeking an explanation for the particular serological and adhesive properties exhibited by the amoebae and spore cells it was found that surface materials obtained by washing cells in 0.5% NaCl were antigenic (ring and precipitin tests) with MA and SA. Such preparations were subjected to two types of analyses to compare quantitatively the amounts of material which had been extracted from the amoebae and spore cell surfaces. These determinations revealed that greater amounts of antigenic material were found in spore wash as compared to amoebae wash (Tables IV, V). To account for the difference in the amount of antigenic material recovered from surface washings of amoebae and spores it is suggested that an increase occurs in the amount of loosely bound surface antigen on the spores. Another possibility would include an elimination of the antigens from the pre-stalk and pre-spore surfaces as a consequence of their role in morphogenetic movements during the culmination process. Antigens ejected from the cell surfaces would be contained in the spore extracellular spaces. A mechanism by which surface antigens are discharged and renewed at the surfaces of the cells would result in the reversibility of cell adhesiveness. Such reversibility is conceivably necessary if cells in contact with each other are to retain their motility.

Upon injecting spore washings into rabbits, antiserum was produced which was capable of agglutinating both amoebae and spores (Table II). Such antiserum (SSA) when subjected to absorption procedures with amoebae or spores reacted with test cells in the same way as the antiserum (SA) produced by injecting whole spores into rabbits (Tables II, III).

The fact that antiserum obtained from whole spore injection cannot be distinguished from spore surface wash antiserum suggests that the same antigens were involved in the production of each antiserum. The ease with which spore surface wash antigen may be removed implies that it is either loosely bound to the cell surfaces or was discharged from the cells during morphogenesis. Therefore, it seems plausible to attribute the poor agglutination of the spores to the inability of the antigens, with which the antibodies combine, to remain attached to the cell surfaces. Consequently, a reduction in the number of antigenic sites involved in effecting agglutination of cells in the presence of antisera would occur. Few antigenic sites on the spore surfaces would probably suffice for the production of antisera in rabbits to those particular antigens, which accounts for the agglutinating ability of the spore antiserum (SA) on amoebae or spores. Another possibility exists that the poor agglutination of the spores might be attributed to the relative inaccessibility of antigenic sites on spore surfaces. Such an interpretation, for the failure of ox red cells to agglutinate in the presence of certain antisera, was advanced by Coombs *et al.* (1951). The existence of this type of cell surface configuration of the spores could conceivably be tested by Coombs *et al.*'s method.

The poor agglutinating capacity of spores is paralleled by weak adhesiveness between the spores. It is interesting to point out that relatively large amounts of antigen appear in washings of spore cell surfaces. These facts suggest that the particular agglutinating and adhesive properties of the spores are related to the appearance of antigen in spore washings.

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SUMMARY

1. Methods are described whereby antigenic material of the slime mold, *D. discoideum*, effective in antibody production in rabbits, is prepared.
2. Antisera produced in response to migrating pseudoplasmodia, mature spores and mature spore surface antigens effected maximal agglutination of amoebae. Such antisera effected only minimal agglutination of spores.
3. Antisera of the above types absorbed by amoebae and spores in all combinations revealed the presence of a surface antigen(s) on the spores which is (are) not common to amoebae from early aggregates or migrating pseudoplasmodia.
4. It has been suggested that a relationship exists between the adhesive properties of the amoebae and spores and their agglutinating properties in the presence of antisera.

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THE EFFECT OF POTASSIUM DEFICIENCY ON THE FREE AMINO ACID PATTERN OF THE MUSCLE TISSUE OF PROTEIN-MAINTAINED *FUNDULUS HETEROCLITUS*^{1, 2}

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In this investigation the effect of a low potassium environment on the free amino acid pattern of the skeletal muscle tissue of the killifish, *Fundulus heteroclitus*, has been studied in an attempt to determine if the changes observed in the potassium-deficient rat (Eckel, Pope and Norris, 1954; Iacobellis, Muntwyler and Dodgen, 1956) could also be found in other members of the vertebrate subphylum. Apparently the rat, but not the dog (Iacobellis, Griffen and Muntwyler, 1957), is capable of replacing part of an intracellular loss of potassium ions with organic cations in the form of basic amino acids, provided a sufficient amount of protein is included in the diet. Amino acids which could serve to restore an intracellular cation-anion balance include lysine, arginine, and histidine. All three carry a net positive charge at body pH. This study, therefore, was made to determine if an intracellular organic cation replacement occurs in potassium-deficient *Fundulus* and, if so, which basic amino acids are responsible for the replacement of the lost potassium.

METHODS

Killifish (*Fundulus heteroclitus*) were trapped in the brackish water of the Oyster River near Durham early in August of 1958 and transported to the laboratory where they were placed in a large aquarium containing artificially prepared 50% sea water (salinity = 18 parts per thousand). The formula followed was taken from the Marine Biological Laboratory at Woods Hole manual, "Formulae and Methods, IV," 1954. After a three-day period of adjustment, one-half of the animals was left in 50% sea water to serve as controls, whereas the other half was placed in an adjacent aquarium also containing 50% artificial sea water but lacking potassium ions. Control and potassium-deficient animals were maintained on a diet of powdered, crude casein.

At the end of a two-week period animals were removed from each aquarium and their tissues prepared for paper chromatography. In order to obtain 10 grams of skeletal muscle tissue in each case it was necessary to sacrifice 7 control *Fundulus* and 8 potassium-deficient *Fundulus*. The fish were skinned and filleted. Ten grams of muscle were macerated in a Waring Blendor, using about 100 ml. of 95% ethanol. After 5 minutes of blending at a moderate speed the liquid was

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transferred to 50-ml. centrifuge tubes and centrifuged for 5 minutes at 3000 r.p.m. The centrifugate was removed (the precipitate consists chiefly of protein) and reduced *in vacuo* to a volume corresponding to 1 ml. per gm. of tissue extracted. Slight heat was applied to speed reduction. While awaiting use, the extract samples were kept in the refrigerator.

Two-dimensional ascending paper chromatography was employed to determine in a semi-quantitative manner the free amino acids present in the muscle tissue of the control and potassium-deficient *Fundulus*. Aliquots of 60 μ l. were spotted on Whatman No. 1 filter paper sheets (9" by 12") and developed for 16 hours with a phenol-water (4:1) solvent. The chromatograms were dried at room temperature and developed for 16 hours in the second dimension with a lutidine-water (2:1) solvent. After drying, the amino acids were located by spraying the chromatograms with a 0.1% solution of ninhydrin in 95% ethanol. The Sakaguchi test for arginine and the Pauli test for histidine were also employed (Block, Durrum and Zweig, 1955). The location of lysine was confirmed on the ninhydrin-developed chromatograms by exposing them to hydrochloric acid fumes for one minute. The spot representing lysine turns a bright yellow (Szantai, 1957) which is stable for several days. Valine, leucine and threonine also turn yellow, but the color soon fades. This, taken in conjunction with the relative positions of the above-named acids on the two-dimensional chromatogram, makes any confusion of valine, leucine or threonine for lysine unlikely.

Total amino acid concentration was determined quantitatively as follows. Ten μ l. of the tissue fluid extracts were spotted on Whatman No. 1 filter paper. Also spotted on the filter paper were 10 μ l. quantities of a standard solution containing 6 μ gm. of amino acid per μ l. Since three amino acids, glycine, alanine and taurine, were found to be responsible for the bulk of the color observed on the ninhydrin-sprayed chromatograms, the standard was prepared by dissolving 20 mg. each of these amino acids in 10 ml. of distilled water. It was felt desirable to use this type of standard to allow for differences in the absorption characteristics of the ninhydrin complexes. After application the spotted amino acid solutions were allowed to dry at room temperature, then were sprayed with a 0.5% solution of ninhydrin in 75% ethanol plus 0.5% 1 N sodium hydroxide. Kay, Harris and Entenman (1956) report that the presence of sodium hydroxide is essential for the development of color in the case of taurine, but otherwise has no effect. Curiously, the author found that sodium hydroxide had no such specific enhancing effect in the case of taurine, although a general increased color density was observed. The ninhydrin-treated filter paper was dried at room temperature and then heated at 60° C. for 25 minutes. The colored areas were cut out and eluted with 5 ml. of a 75% ethanol solution containing 10 mg. of cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in 200 ml. of solution (Giri, Radhakrishnan and Vaidyanathan, 1952). Blanks were prepared by cutting out portions of filter paper equal in area to those supporting the colored complexes. Optical density of the colored solutions was read at 540 $m\mu$ on a Bausch-Lomb "Spectronic 20."

Two-dimensional paper chromatography was used to determine the amount of lysine present in the muscle tissue of potassium-deficient and control *Fundulus*. Sixty- μ l. aliquots were used in the case of the potassium-deficient fish whereas 400 μ l. were required in the case of the control animals, due to the very low con-

centration of lysine in their tissues. Three to 5 μ l. of an aqueous solution containing 6 mg. of lysine per ml. of solution, serving as standards, were spotted and chromatographed. Chromatograms were developed using phenol-water (4:1) and lutidine-water (2:1) as solvents. After drying at room temperature the chromatograms were sprayed with ninhydrin as before. Apparently the spraying procedure is the most crucial point in the quantitative determination. Care should be taken to spray the chromatograms lightly and evenly on one side, allow them to dry, then repeat the operation on the other side. The chromatograms were heated, lysine spots cut out and eluted and optical density read as in the case of the determination of total amino acid concentration.

RESULTS

Fifteen amino acids were found to be present in the extracts of the control and potassium-deficient animals. The same amino acids were present in both cases. Furthermore, it was found that (1) the total amino acid concentration

TABLE I

The total concentration of amino acids and the concentration of lysine in the skeletal muscle of control and potassium-deficient Fundulus heteroclitus

	Total concentration (in mg./gm. of tissue)		Lysine concentration (in mg./gm. of tissue)	
	Potassium- deficient <i>Fundulus</i>	Control <i>Fundulus</i>	Potassium- deficient <i>Fundulus</i>	Control <i>Fundulus</i>
Range	4.9–6.2	2.7–3.3	0.32–0.42	0.030–0.035
Mean	5.4*	3.0*	0.38†	0.033†
No. of determinations	13	17	4	3

* Difference is significant at 0.1% level.

† Difference is significant at 1.0% level.

in the tissues of the potassium-deficient *Fundulus* appeared to be somewhat greater than in the controls (Table I), and (2) the concentration of lysine in the potassium-deficient fish was much greater than in the controls, whereas the levels of histidine and arginine did not seem to be disproportionately large in the former. It was felt that the apparently greater total amino acid concentration observed in the potassium-deficient animals could not account for the extreme differences seen in the concentration of lysine.

Table I shows the values obtained from 17 determinations of the total amino acid concentration in the tissues of the controls and 13 determinations of the potassium-deficient fish. A certain amount of variation occurs in both cases. However, the extent of variation is relatively small when the difficulty of the method is considered. As was suggested by the semi-quantitative determination, a significantly greater concentration of free amino acids occurs in the muscle tissues of the potassium-deficient *Fundulus*. Although in the controls the concentration

averages 3.0 mg. per gm. of tissue extracted, it reaches a level of 5.4 mg. per gm. in the potassium-deficient forms, *i.e.*, a concentration 1.8 times as great.

Table I indicates the values obtained for lysine levels in control and potassium-deficient *Fundulus*. Although only three determinations were made on the control animals and four on the potassium-deficient animals, the degree of variation was small in both instances and the differences observed in the lysine concentrations are regarded as significant. Indeed, the concentration of lysine in the skeletal muscle of the potassium-deficient forms appears to be more than 10 times as great as that of the control animals, whereas the total amino acid concentration is only 1.8 times as great.

DISCUSSION

If animals are made deficient in potassium an influx of available extracellular cations would be expected in order to restore the cation-anion balance. In certain animals the lost potassium can be partly replaced by a gain in sodium. According to Cooke and his co-workers (1952) the remainder is replaced by an intracellular migration of protons, the result being the development of a plasma alkalosis. However, several investigators (Muntwyler, Griffen and Arends, 1953; Iacobellis, Muntwyler and Dodgen, 1956; Holliday and Segar, 1957) observed no plasma alkalosis in the potassium-deficient rat. Since Christensen, Riggs and Palatine (1952) showed that basic amino acids could act as intracellular cations, Eckel and his co-workers (1954) investigated the possibility of this being the case in potassium-deficient rats demonstrating no plasma alkalosis. Their results revealed that 8 to 40% of the intracellular metallic cation deficiency is replaced by lysine.

The results of this investigation show that, as in the case of the rat, the cation-anion imbalance appearing in the potassium-deficient *Fundulus* is partially overcome by an increase in the intracellular concentration of lysine. In Table I it can be seen that the concentration of lysine in the potassium-deficient *Fundulus* is approximately 10 times greater than in the controls. In terms of per cent of total amino acid concentration, the value for lysine is 1.1% in the case of the control animals and 7.0% in the potassium-deficient animals, an increase greater than 500%.

The present results indicate that histidine and arginine contribute little to the increase in intracellular organic cation concentration in *Fundulus*. Furthermore no decrease was observed in aspartic and glutamic acids as Iacobellis, Muntwyler and Dodgen (1956) found with Wistar rats.

The total free amino acid concentration in the skeletal muscle tissue of potassium-deficient *Fundulus* is almost twice that of the controls (Table I). The observed increase may be the result of a decrease in the rate of nitrogen anabolism and perhaps the establishment of a negative nitrogen balance. Evidence that such is the case here is supplied by the work of Muntwyler, Griffen and Arends (1953) and Eckel, Norris and Pope (1958). Both groups reported decreased growth and a failure to maintain a proper nitrogen balance in the potassium-deficient rat. Conceivably in *Fundulus* as well as the rat an increased amount of free amino acids would be due to a failure of body protein synthesis and in certain cases (lysine) could serve to ameliorate a state of cation-anion imbalance by functioning as intracellular cations.

The fact that an identical device for combatting a loss of cellular potassium can be seen in animals as widely separated as mammals and fish suggests the

interesting possibility of the universality of such a mechanism. It may be that a similar or perhaps identical mechanism accompanying a potassium deficiency can be found throughout the entire vertebrate group, indeed, perhaps in several phyla of the animal kingdom.

SUMMARY

1. The effect of a potassium deficiency on the free amino acids of the muscle tissues of *Fundulus heteroclitus* was investigated employing the method of quantitative paper chromatography.

2. An over-all increase in the total amino acid concentration was observed in the potassium-deficient fish amounting to 1.8 times that seen in the controls. A great increase in the concentration of lysine was also observed in the potassium-deficient forms. In this case the concentration was about 10 times that of the controls.

3. It is therefore suggested that the mode of combating a potassium deficiency in the rat also exists in a fish. The interesting possibility that similar or perhaps identical mechanisms for lessening the effects of a potassium deficiency may be found throughout other representative vertebrate groups, as well as the invertebrates, offers a fruitful field for further comparative investigations.

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STRUCTURAL AND FUNCTIONAL CHANGES IN A GENERATION IN TETRAHYMENA¹

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With the development of techniques for synchronizing the division of members of a population of microorganisms, it has become possible to analyze many changes that occur in a cell during a generation (the period from the end of one division to the end of the next). The ciliate protozoan *Tetrahymena pyriformis* has been used in this way by Zeuthen and his coworkers in studies directed at biochemical changes; nitrogen content (Hamburger and Zeuthen, 1958), nucleoside triphosphate concentration (Plesner, 1958a), rate of incorporation of P³² (Hamburger and Zeuthen, 1958), and sensitivity to anaerobiosis (Rasmussen, 1958), dinitrophenol (Hamburger and Zeuthen, 1957), and inhibitors of oxidative metabolism (Hamburger, 1958). Synchronously-dividing populations are also useful for tracing the time course of structural changes. *Tetrahymena* is uniquely suited to such a study since it passes through a period of morphogenesis in each generation that results in duplication of the structures necessary for the formation of a new individual before division (body cilia, mouth, contractile vacuoles, cytopyge) (Chatton *et al.*, 1931; Furgason, 1940), and it undergoes nuclear changes involving a mitotic division of the micronucleus and an amitotic division of the macronucleus (Maupas, 1883, 1888).

The following is a description of the time course of the structural changes (those visible with the light microscope) in a generation, in a synchronized population of *Tetrahymena pyriformis*, mating type I, variety 1, and a comparison of it with the time course of known chemical changes in a generation, observed with other strains of *Tetrahymena*.

MATERIALS AND METHODS

Division was synchronized by the application of 5 alternate 1½-hour exposures to temperatures of $42.6 \pm 0.2^\circ \text{C}$. and $35 \pm 0.2^\circ \text{C}$. Other details of procedure were as described by Holz, Scherbaum and Williams (1957). Following the final high temperature period, samples of ciliates were withdrawn at 5-minute intervals for 180 minutes. To determine when the first and second synchronous divisions occurred, the division index (cells in division ÷ cells counted) was measured for each sample. The first synchronous division (70–80% synchrony) occurred 50–60 minutes after the last high temperature period, and the second division (45–55% synchrony) 65–75 minutes after the first division. Changes in the nuclear apparatus (Fig. 1), infraciliature (Fig. 8), and general appearance of the ciliates during the interval between divisions were followed by Feulgen-staining (Dippell

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and Chao's modification of the DeLamater stain; Sonneborn, 1950), silver impregnation (Chatton-Lwoff technique; Corliss, 1953), and the observation of living individuals with a phase contrast microscope. The temporal positions of the nuclear and morphogenetic events in a generation were determined by comparisons of the times of greatest frequency of appearance of these events (in the samples taken between the first and second synchronous divisions) with the times when the first and second synchronous divisions occurred.

To establish a basis for comparison of the times of structural changes in mating type I, variety 1, with the times of changes in physiological and biochemical parameters reported for other strains, all generation times of the various strains used were compressed to 100 arbitrary units and the time courses of all changes adjusted to fit this scale. For synchronously-dividing cells, the generation time used in these calculations was that of the synchronous generation during which observation of a particular structural or functional change was made. With one exception this was the second synchronous generation (the period between the first and second synchronous divisions). Nucleoside triphosphate content was determined at various times during the first synchronous generation (the period from the end of the last high temperature exposure to the first synchronous division) (Plesner, 1958a). Data on DNA synthesis (McDonald, 1958) and sensitivity to extremes of temperature (Thormar, 1956) were obtained with single normal cells, nitrogen content (Hamburger and Zeuthen, 1958), sensitivity to dinitrophenol (Hamburger and Zeuthen, 1957), sensitivity to inhibitors of oxidative metabolism (Hamburger, 1958), nucleoside triphosphate content (Plesner, 1958a), and rate of incorporation of P^{32} (Hamburger and Zeuthen, 1958) were measured with groups of synchronized cells, and rate of respiration (Zeuthen, 1953; Zeuthen and Scherbaum, 1954) and sensitivity to anaerobiosis (Rasmussen, 1958) with both types. Such a comparison is based on several assumptions: first, that there are no gross differences in the time scales of structural and functional events in a generation in different strains of *Tetrahymena pyriformis* with different generation times and nuclear constitutions (micronucleate and amiconucleate), *i.e.*, that each event occurs at the same relative time in every strain; second, that the time characteristics of structural and functional changes occurring between synchronous divisions can be compared with those occurring between divisions of normal cells (cells that have not been heat-treated).

The first assumption is made on the basis that the structural and chemical changes that have been studied are of such a fundamental nature, necessary to the orderly progression of growth and preparation for division, that they probably follow the same relative time courses in all strains. This assumption was tested experimentally, so far as structural changes were concerned, by comparing the time courses of morphogenetic and macronuclear changes in strain GL and in mating type I, variety 1, with synchronized populations. Strain GL was synchronized by the method of Scherbaum and Zeuthen (1954). Comparison was also made of the time course of structural changes of mating type I, variety 1, with that reported by Browning, Varnedoe and Swinford (1952) for strain T-P. McDonald (1958) and Prescott and Bors (1958), using different strains, reached fundamentally the same conclusion concerning the period of DNA synthesis in a generation.

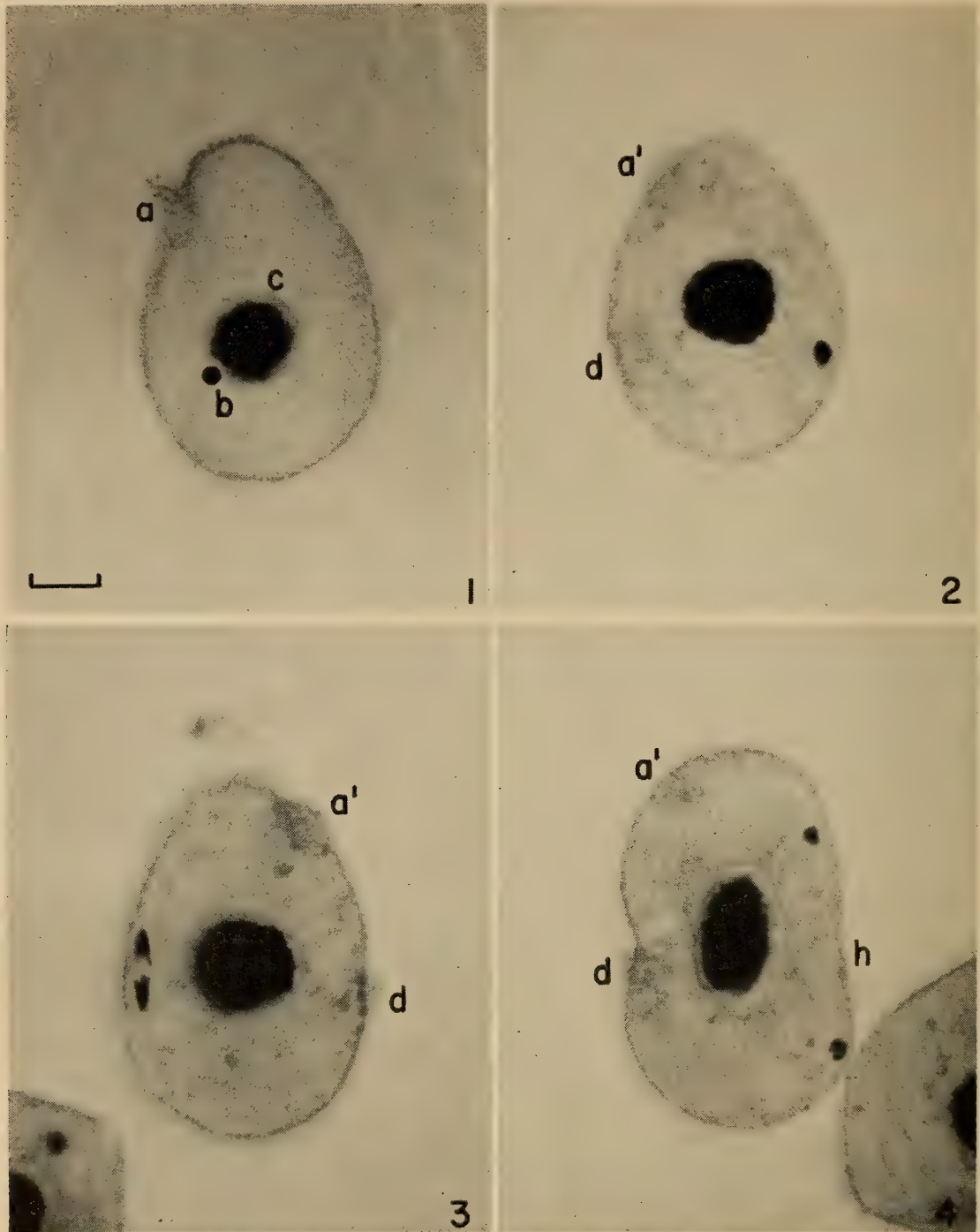


FIGURE 1. Resting nuclear configuration in *Tetrahymena pyriformis*, mating type I, variety 1. In this and the following illustrations of nuclear states, ciliates were not destained to a degree necessary to bring out fine nuclear detail. This was done to insure visualization of the cilia of the mouth, to assist in observing time relations of nuclear and morphogenic states. The marker indicates $10\ \mu$ for Figures 1-7.

FIGURE 2. Stage of macronuclear enlargement. The micronucleus is peripherally located. Cilia of the forming mouth of the opisthe are seen.

FIGURE 3. Micronuclear anaphase.

FIGURE 4. Micronuclear division complete. Macronuclear division and cytoplasmic cleavage beginning.

The second assumption is more open to question. Cells prepared for synchronous division by the temperature-cycling method have a shorter generation time (Zeuthen and Scherbaum, 1954), have a greater volume (Scherbaum, 1956), greater reduced weight (Scherbaum and Zeuthen, 1954), greater dry weight (Scherbaum, 1957b), and contain more nitrogen (Zeuthen and Hamburger, 1956), DNA and RNA (Scherbaum, 1957b), ATP and GTP (Plesner, 1956, 1958b) than do normal cells. During the synchronous divisions these characteristics return to normal. To liken the time courses of biochemical changes that have been studied in these cells to the condition in normal cells, we must assume that the differences noted above influence only the extent of the changes studied and not their time characteristics with respect to division. In this connection, it is notable that rate of respiration (Zeuthen, 1953; Zeuthen and Scherbaum, 1954) and sensitivity to anaerobiosis (Rasmussen, 1958) follow the same time courses between divisions in heat-treated and normal cells. The chemical composition of the heat-treated cells might cause the morphogenetic and nuclear changes to follow different time courses *with respect to one another*. To test this possibility, synchronized cells, and normal cells from cultures reproducing at an exponential rate, were compared for nuclear state in silver impregnations showing stages of stomatogenesis. In such preparations the micronucleus shows up well except during its final division stages.

RESULTS

The resting micronucleus was spherical and lay next to the macronucleus, or within a cup-shaped depression in the macronucleus (Fig. 1). The resting state was assumed immediately after completion of cytoplasmic cleavage and lasted for about 75% of a generation. At the end of this period the micronucleus moved laterally toward the periphery of the organism and elongated (Fig. 2). Chromosomes appeared at this time. The most clearly recognizable stages of mitosis were those associated with separation of the chromosomes (Fig. 3). As the chromosomes reached the poles of the division figure the latter greatly elongated and the micronucleus was divided into halves which became separated respectively into the anterior and posterior parts of the dividing organism (Fig. 4). These daughter micronuclei then became spherical again. The division of the micronucleus went to completion as macronuclear division and cytoplasmic cleavage began.

KEY TO FIGURES 1-16

- a Mouth of "resting" (interphase) ciliate.
- a' Mouth of proter (anterior daughter) during stomatogenesis.
- b Micronucleus.
- c Macronucleus.
- d Mouth of opisthe (posterior daughter).
- e Macronuclear fragment separated during macronuclear division.
- f Stomatogenous field, site of formation of mouth of opisthe.
- g Contractile vacuole pore of proter.
- h Cleavage furrow.
- i Stomatogenous meridian.
- j Formation of membranelles.
- k Formation of undulating membrane.

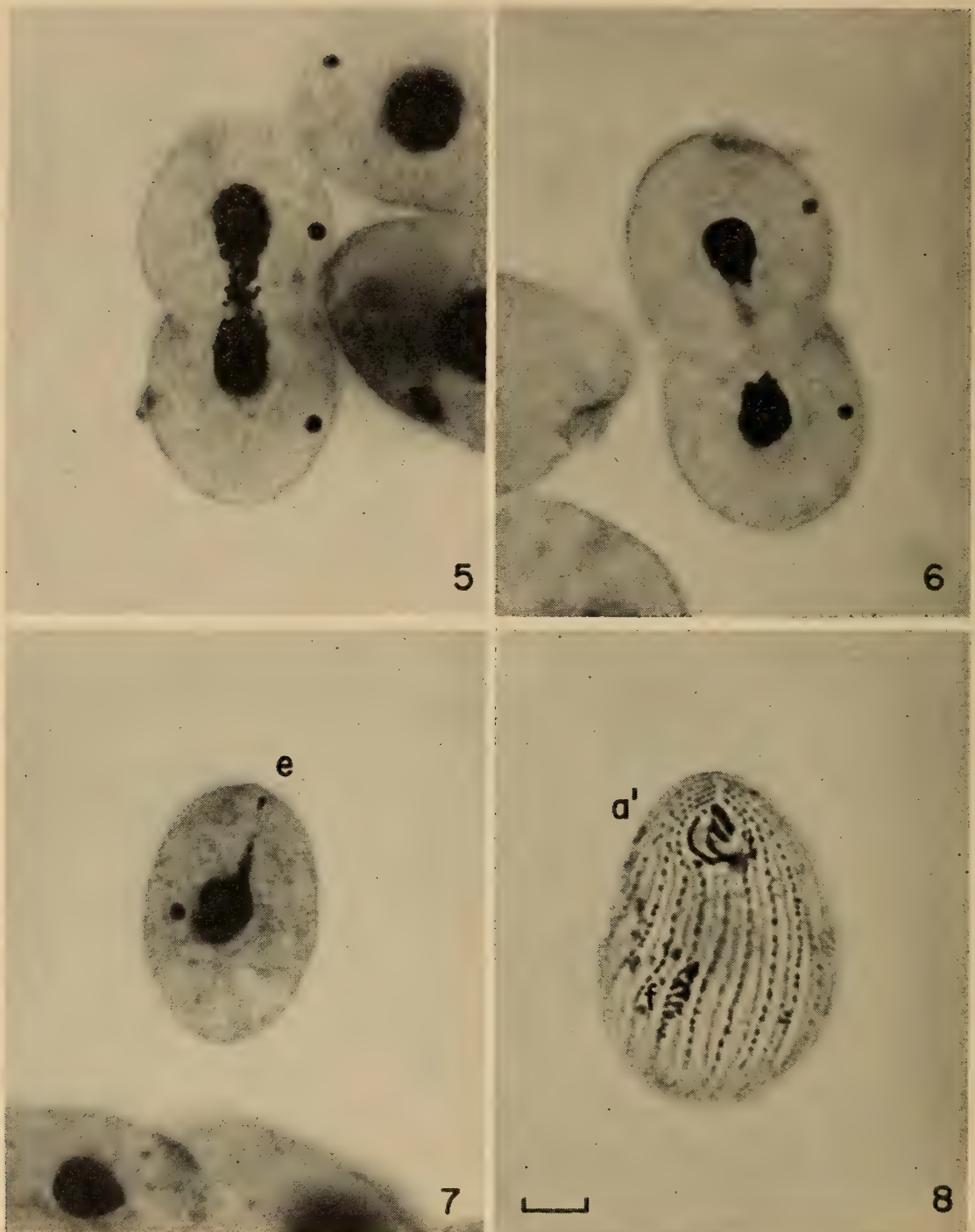


FIGURE 5. Macronuclear division and cytoplasmic cleavage continuing.

FIGURE 6. Macronuclear division complete. Cytoplasmic cleavage continuing.

FIGURE 7. Product of a recent division, showing re-establishment of resting nuclear configuration.

FIGURE 8. Infraciliature of *Tetrahymena pyriformis*, mating type I, variety 1. Note normal mouth of proter with typical tetrahymenal configuration, and stomatogenesis to produce mouth of opisthe. The marker indicates 10 μ for Figures 8-11.

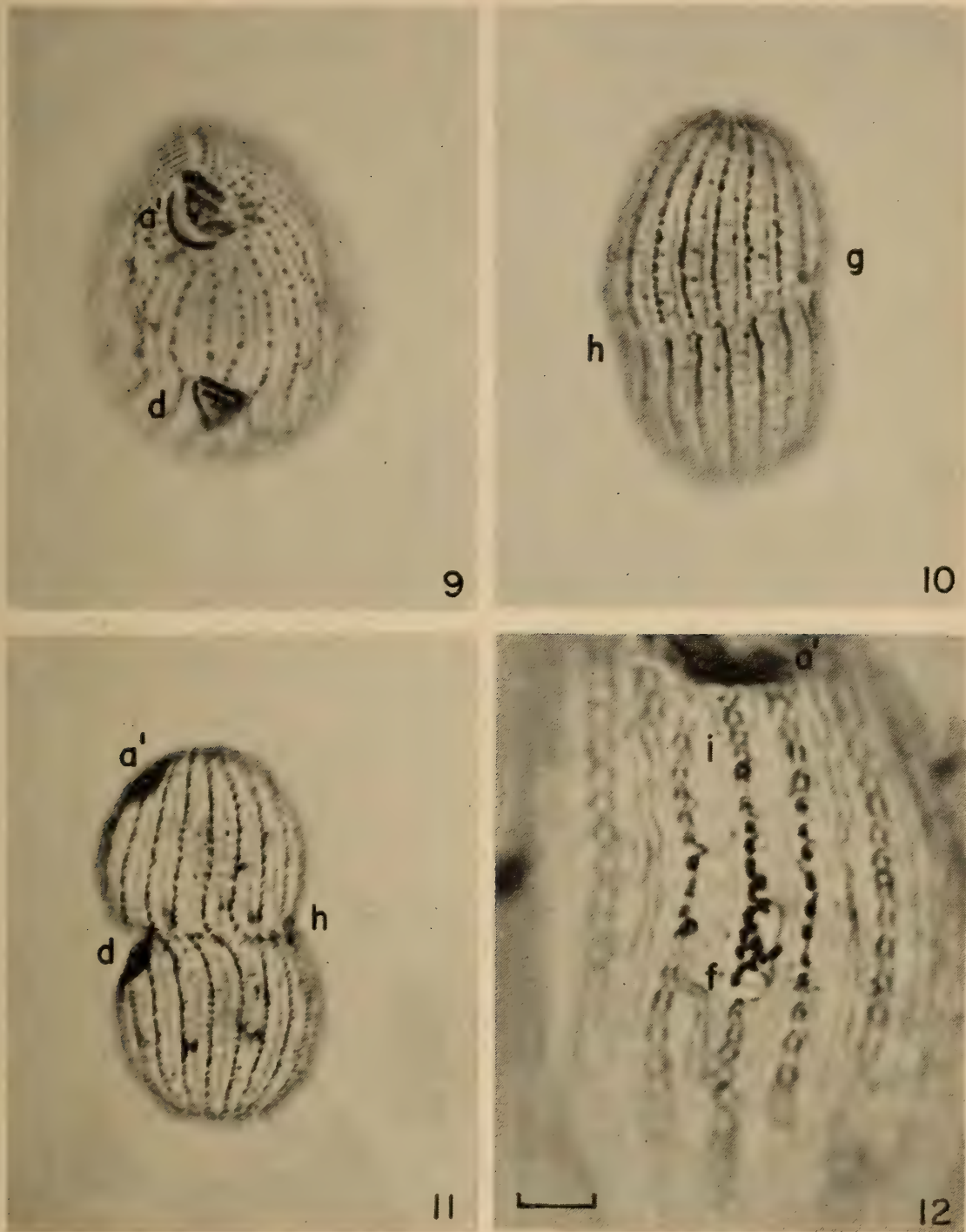


FIGURE 9. Completion of stomatogenesis in opisthe.

FIGURE 10. Appearance of cleavage furrow at beginning of division (dorsal surface of ciliate). Note contractile vacuole pore of opisthe just anterior to furrow on right side of animal.

FIGURE 11. Cleavage.

FIGURE 12. Very early stage of stomatogenesis. Kinetosomes appear to left (animal's left) of stomatogenous meridian. The marker indicates $5\ \mu$ for Figures 12-16.

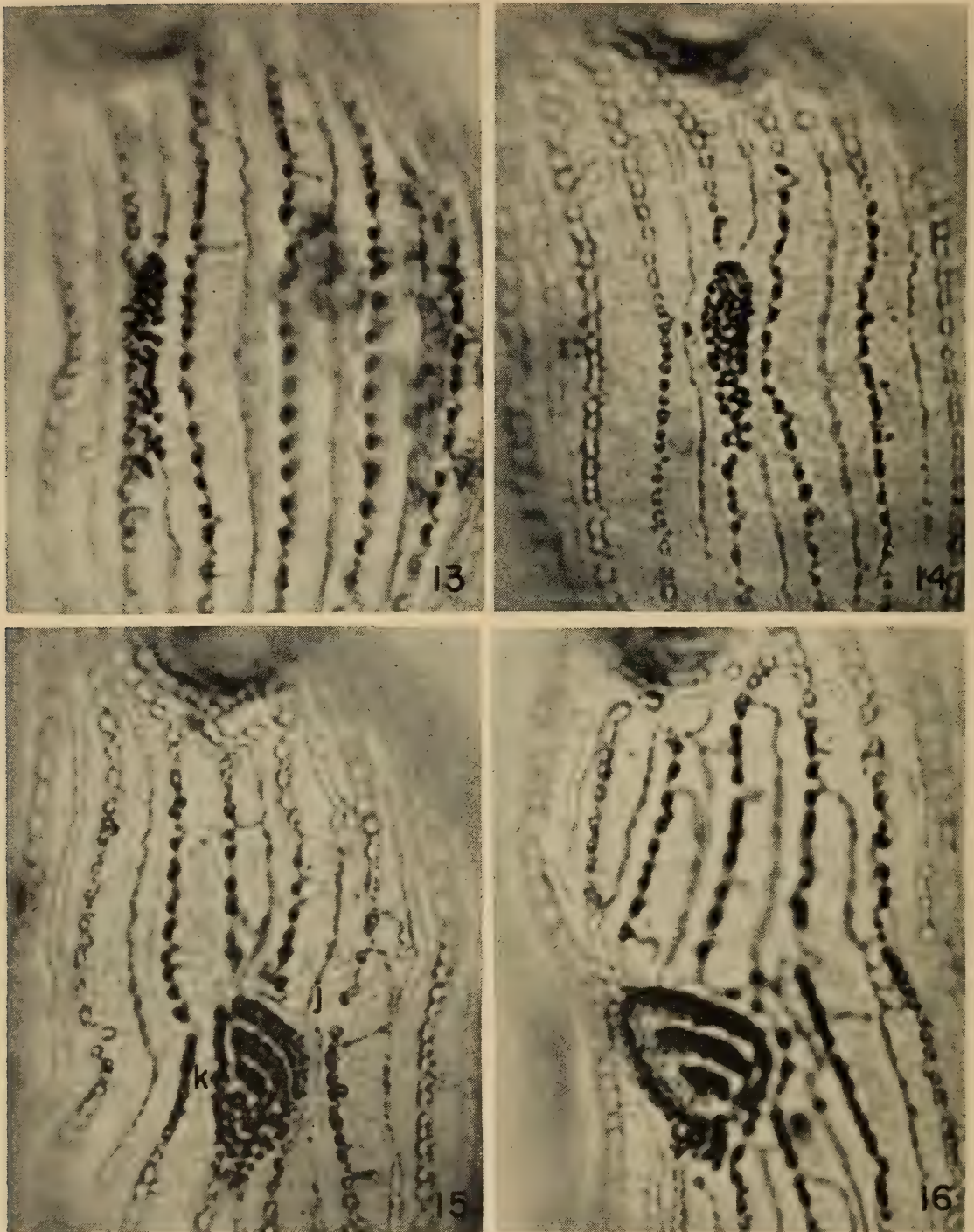


FIGURE 13. Stomatogenesis; anarchic field.

FIGURE 14. Stomatogenesis. Very early stage of membranelle organization at anterior end of field.

FIGURE 15. Stomatogenesis. Three membranelles and undulating membrane formed.

FIGURE 16. Stomatogenesis; terminal stage.

The macronucleus was an approximately spherical, centrally-located structure (Fig. 1). Immediately after cleavage, it assumed a resting state characterized by a moderately dense and homogeneous granular appearance. At the time the micronucleus migrated to the periphery of the organism, the macronucleus in-

creased in size and granularity (Fig. 2). As the micronucleus divided, the macronucleus elongated (Fig. 4), and at the time the cleavage furrow separated the daughter ciliates, it divided into approximately equal halves (Figs. 5 and 6). Small portions of the macronucleus occasionally separated from the main macronuclear masses at this time and became isolated in the cytoplasm as spherical, Feulgen-positive bodies which were later resorbed (Furgason, 1940; Scherbaum *et al.*, 1958).

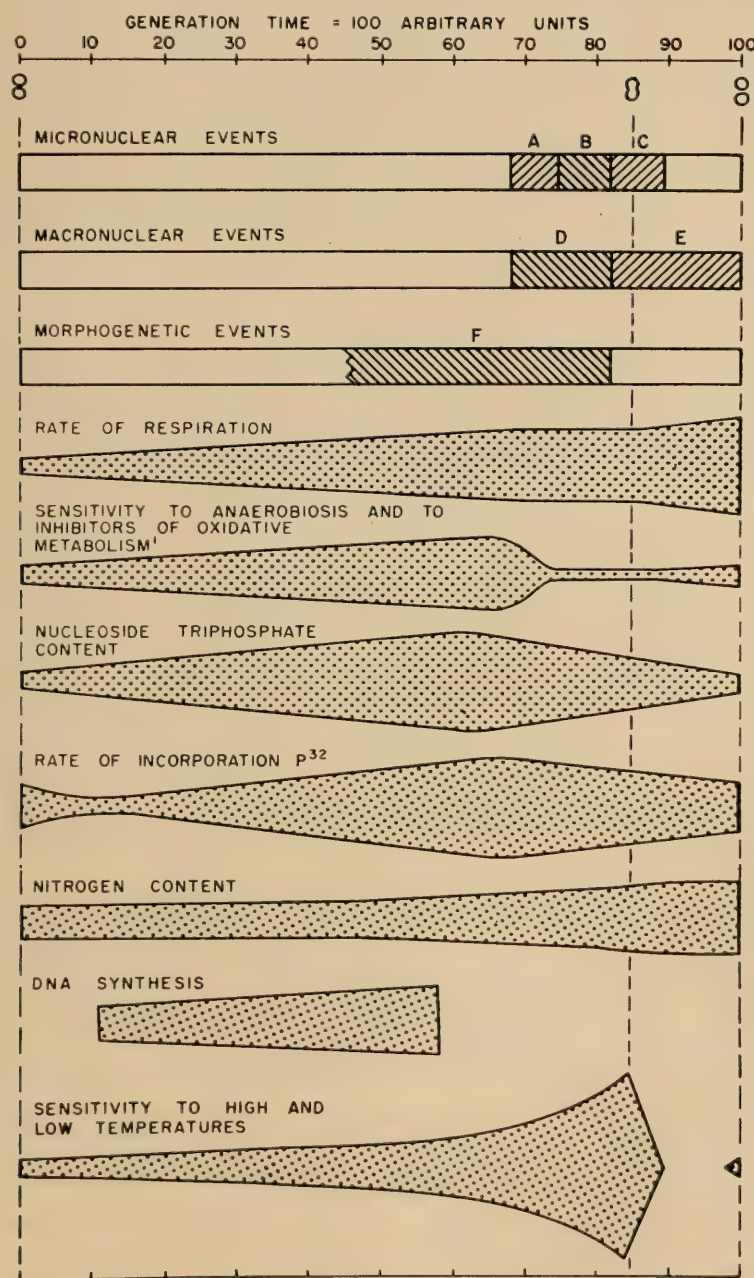


FIGURE 17. Graphic representation of approximate time relationships of structural and functional changes occurring in *Tetrahymena pyriformis* in a generation. (A—migration of micronucleus to periphery, B—mitosis, C—separation of products of mitosis into daughters, D—macronuclear swelling, E—macronuclear division, F—stomatogenesis, 1-nitrogen; Rasmussen, 1958, dinitrophenol; Hamburger and Zeuthen, 1957, fluoride, fluoracetate, azide, ethylurethane; Hamburger, 1958).

Morphogenesis in *Tetrahymena* involves formation of new body cilia, a new mouth (stomatogenesis) for the posterior daughter (opisthe), and new contractile vacuole pores and a cytopye (cell anus) for the anterior daughter (proter). Body cilia are duplicated at a time not yet precisely determined. Stomatogenesis (mouth formation) was manifested first by the appearance of kinetosomes (basal bodies of cilia) to the left of the stomatogenous kinety (row of cilia, or primary ciliary meridian, passing posteriorly from the right side of the buccal overture) near the middle of the body (Fig. 8). The precise time when stomatogenesis began was not determined but early stages were seen before 45% of a generation was completed. Successive stages in the duplication of the kinetosomes of the cilia of the new mouth and their organization into membranelles are shown in Figures 12-16. The anlage of the mouth was in the late anarchic field stage (Fig. 14), or early stage of membranelle organization, at the time the micronucleus assumed a peripheral position. Stomatogenesis was complete, and the membranelles of the mouth appeared to be functional in living ciliates at the time of division of the micronucleus (Figs. 9, 15, 16).

The contractile vacuole pores of the proter were seen in silver impregnations (Fig. 10) as early as the time of lateral movement of the micronucleus, and the contractile vacuole was observed functioning in living organisms at this time.

The cleavage furrow first appeared just anterior to the mouth of the opisthe at the time of separation of the halves of the dividing micronucleus. A space appeared between kinetosomes of the primary ciliary meridians, in a line around the equator of the organism (Fig. 10). The furrow deepened until the proter and opisthe were separated. Members of the genus *Tetrahymena* should be excellent organisms for testing theories of the mechanism of cleavage since they have markers, the kinetosomes, in the cortex. An examination of the pattern of infraciliature in the cortex during cleavage revealed no distortion except in the region of the cleavage furrow, where the cilia-free space appeared (Fig. 10), and where a slight torsion of the ends of the kineties next to the furrow sometimes occurred (Fig. 11). Theories which depend upon polar expansions (active or passive) of the cortex accompanying furrow formation (Swann and Mitchison, 1958) seem to be untenable for *Tetrahymena*.

Except for the absence of the micronucleus in strains GL and T-P, no differences in the relationships *between* nuclear and morphogenetic events were noted when normal cells of strain GL and mating type I, variety 1, were compared, when normal and synchronized cells of mating type I, variety 1, were compared, or when normal cells of strain T-P and mating type I, variety 1, were compared (data of Browning *et al.*, 1952).

Figure 17 is a graphic representation of the approximate time relationships of various structural and functional changes occurring in *Tetrahymena pyriformis* in a generation.

DISCUSSION

As noted by Zeuthen (1958), there are striking associations between those biochemical changes related to energy production, and the initiation and completion of nuclear events associated with division and the process of cytoplasmic cleavage. Rate of respiration, sensitivity to anaerobiosis and to dinitrophenol and inhibitors of oxidative metabolism, nucleoside triphosphate content, and rate of P^{32} incorpora-

tion reach a maximum at the time of initiation of nuclear events. During the following period of nuclear changes, before the appearance of the cleavage furrow, the rate of respiration remains high and constant, sensitivity to anaerobiosis and to dinitrophenol and inhibitors of oxidative metabolism are at a minimum, and nucleoside triphosphate content and the rate of incorporation of P^{32} decline. When the cleavage furrow appears, sensitivity to anaerobiosis and to dinitrophenol and inhibitors of oxidative metabolism begins to rise, while nucleoside triphosphate content and the rate of P^{32} incorporation continue to decline. As cleavage proceeds, the rate of respiration begins to rise again.

The last 25% of a generation is also a period that shows the greatest fluctuations in sensitivity to extremes of temperature. Sensitivity is at a minimum during the final $\frac{2}{3}$ of cytoplasmic cleavage. It begins to rise just before the completion of cleavage and rises gradually until the time of nuclear changes, when it rises abruptly to reach a maximum just before the onset of visible cleavage. It then drops sharply to a minimum during the first $\frac{1}{3}$ of cleavage (Thormar, 1956). The most sensitive time coincides with the time of chromosome movement and the elongation of the micronucleus accompanying its division, just before the macronucleus begins to elongate, and at the time of final organization of the membranelles of the mouth of the opisthe. These nuclear and morphogenetic stages are characteristic of ciliates prevented from dividing by the cyclic heat treatments used to induce synchronous division (Holz *et al.*, 1957). The high sensitivity is not attributable to the effects of temperature on micronuclear division, since the amicro-nucleate strain GL seems to be blocked at the same point in time (Williams and Scherbaum, 1959). It is not possible at present to associate the high sensitivity of this period with any *one* of the coincident processes occurring (nuclear changes, morphogenetic changes, preparation for cytoplasmic cleavage). The cyclic heat treatments do not prevent the early stages of mitosis, amitosis or stomatogenesis, but do stop these processes at very characteristic stages and have no differential effect on them such that one progresses in time beyond the others. In normal cells from cultures which are reproducing at an exponential rate, nuclear changes and stomatogenesis also follow parallel courses, with time characteristics such that the early stages of micronuclear division, just prior to macronuclear elongation, and the late anarchic field stage and early stages of membranelle organization coincide. The fact that mitosis, macronuclear division and stomatogenesis are blocked by high temperature at characteristic stages suggests that the heat-labile biochemical system (systems) postulated by Scherbaum (1957a) and by Zeuthen (1958) is (are) necessary to the completion of all these processes, or that each process is dependent upon the others for its normal progression and goes to completion only when the others are unimpaired.

McDonald (1958), using a Feulgen microspectrophotometric method on the macronuclei of single cells, observed that DNA synthesis began after passage of about 10% of a generation, continued for approximately 45%, and ended shortly before the initiation of nuclear structural changes. In a recent preliminary note Prescott and Bors (1958) report a slightly shorter period of synthesis starting at about the same relative time and ending midway in the generation. They followed the course of synthesis by autoradiographic detection of the incorporation of tritiated thymidine. These findings are not in agreement with the earlier report

of Walker and Mitchison (1957) that a linear synthesis occurs throughout interphase. Duplication of DNA in the first half of a generation is not characteristic of all ciliates. *Euplotes eurytomus* (Fauré-Fremiet *et al.*, 1957), *Paramecium caudatum* (Walker and Mitchison, 1957), and *Paramecium aurelia* (Kimball and Barka, 1959) synthesize their DNA during the last half of a generation.

The author wishes to acknowledge with thanks the helpful suggestions of Dr. Verner Wulff and Dr. Eric Zeuthen.

SUMMARY

The time courses of structural events occurring in a synchronous generation of *T. pyriformis*, mating type I, variety 1, were compared with one another and with the time courses of functional events in a synchronous generation, and/or in a generation of single, normal ciliates, of other strains of the species.

NOTE ADDED IN PROOF

Since this manuscript was accepted for publication two important relevant papers have appeared. Scherbaum *et al.*, *J. Cell. Comp. Physiol.*, **53**: 119–138, 1959, reported that in strain GL the pattern of protein amino acids at stages in normal growth, and during the first synchronous generation of heat-treated cells, was stable. The free amino acids present at the same stages varied in their concentrations, but not in a manner that could be correlated with any of the cytological changes that occurred. Scherbaum *et al.*, *Exp. Cell Res.*, **18**: 150–166, 1959, reported that in strain GL, during the first synchronous generation (end of heat-treatment to onset of first synchronous division), the dry weight, volume, acid-soluble phosphates, acid-insoluble heat-labile phosphates, and DNA content of the average cell increased, and thymidine incorporation into DNA could be demonstrated. In addition, circumstantial evidence from microspectrophotometric experiments, indicated a burst of DNA synthesis just prior to and during the first synchronous division. As the authors suggested, substantiation of this finding would make it unwise to apply information on DNA synthesis obtained with synchronized cells to the process in normal cells.

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ANTIGENS OF THE SEA URCHIN SPERM SURFACE¹

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Morphological and physiological evidence indicates that the initial steps in fertilization involve the sperm and the egg surfaces. Furthermore, these steps would appear to be chemical interactions between surface substances. Beginning with the pioneer studies of F. R. Lillie (1913, 1914), the chemical relationships have been visualized as between complex molecules; and antigen-antibody systems have served as useful models (see Tyler, 1948). Most of the present information has been obtained from studies on agents extracted from sperm and eggs. Notable among these agents are the sperm isoagglutinin, fertilizin, obtained from the egg jelly layer and the egg agglutinating antifertilizin obtained from sperm. Although these and other agents have been studied extensively, their role in fertilization has not been clearly defined (see Metz, 1957a, for review). On the other hand, relatively little effort has been directed toward analysis of the intact cell surface. Contributions in this direction include Tyler's (1946) study of the effect of specific antiserum on the fertilizing capacity of sea urchin sperm. In this study non-agglutinating, univalent antibody prepared against sperm antifertilizin was found to reduce the fertilizing capacity of sea urchin sperm. In an analogous investigation on *Paramecium*, antiserum was found to block initial steps in the mating process (Metz, 1954). Extension of this line of study would seem to promise significant information for an understanding of the interaction of egg and sperm surfaces at fertilization. As a step in such an investigation it seemed desirable to map out the antigenic structure of the *Arbacia* sperm surface. Accordingly, in the present study an effort was made to determine the number of sperm surface antigens and their distribution with respect to the morphology of the sperm, and to examine for sperm surface antigens that do not appear in sea water extracts.

Studies on the antigenic structure of sperm are found in publications extending over a period of 60 years. However, most of such studies were not directed primarily toward an understanding of the fertilization process. More recently Mudd and Mudd (1929), Henle (1938), Snell (1944), Smith (1949) and Pernot (1957) among others have demonstrated species, strain and tissue specificities. Certain of these studies, notably those of Henle *et al.* (1938) and Pernot (1957), are also concerned with the number and distribution of sperm antigens. The former study showed three agglutinogens on bull sperm, one confined to the tails, one restricted to heads and the third common to both. Pernot's (1957) agar diffusion and immunoelectrophoretic study of guinea pig material revealed eleven antigens in

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the seminal plasma and seven in alkaline sperm tail extracts. Six antigens were common to both materials. Pernot also presents some evidence for an antigen common to both heads and tails, and an experiment suggesting another antigen restricted to the guinea pig sperm tail. It remains to be shown which if any of the sperm antigens in Pernot's extracts are components of the cell surface. Finally, Dallam and Thomas (1953) have isolated lipoprotein from sperm heads of several mammalian species. Antisera prepared against such lipoprotein agglutinated sperm.

These studies concern mammalian material. Among echinoderms extensive serological investigations of the egg have been made by Tyler and Brookbank (1956a, 1956b), and the Perlmanns (Perlmann, 1957; Perlmann and Perlmann, 1957a, 1957b; see Runnström *et al.*, 1959, for review). Studies of the sperm include a brief reference by Perlmann (1957) and accounts by Tyler and O'Melveny (1941) and Tyler (1946, 1949). In the last report Tyler (1949) lists cross-agglutination reactions between antiserum and sperm of several species and demonstrates a minimum of two antigens on the *Strongylocentrotus purpuratus* sperm surface.

MATERIAL AND METHODS

Seven echinoderms were employed in the study. These include *Arbacia punctulata*, *Lytechinus variegatus*, *Mellita quinquiesperforata* and *Plagiobrissus grandis* from the vicinity of the Florida State University Marine Laboratory at Alligator Point, Florida, and *Arbacia punctulata*, *Echinarachnius parma* and *Asterias forbesii* from Woods Hole, Mass.

Gametes were obtained from *Arbacia* by electrical stimulation (see Harvey, 1956) or by excising the gonads. Treatment with isotonic KCl was avoided since this method causes release of the antigen-containing dermal secretion (Metz, 1959). Gametes were obtained from *Lytechinus*, *Mellita*, *Echinarachnius* and *Plagiobrissus* by treating the gonads with isotonic KCl.

Sperm and seminal plasma were separated by low speed centrifugation. The sperm was re-suspended once in sea water and centrifuged again. The supernatant was discarded and the settled sperm was made up as a 25% suspension by addition of three volumes of sea water. This stock suspension was used for injections and absorptions. For spot plate agglutination tests a 1% sperm suspension was prepared by diluting the stock suspensions to $\frac{1}{25}$ with sea water.

Injections. To obtain antisera five rabbits were injected with *Arbacia* sperm (25% washed sperm in sea water) or sperm extracts, three rabbits were injected with *Lytechinus*, one with *Echinarachnius* and three with *Asterias* sperm. The immunizing antigens were administered through intravenous, intraperitoneal and subscapular injections. In the last instance the antigen was injected as an emulsion in Freund's adjuvant (Difco). This yielded the antisera with the highest sperm agglutinin titers. Most of the rabbits received two or three courses of injections.

Although anti-*Arbacia* sera from several rabbits were examined with uniform results only experiments with antiserum from the hyper-immune rabbit "#33" are recorded here. This rabbit was injected with antigen in Freund's adjuvant. The animal was bled two weeks subsequent to the injection. The immune serum obtained regularly agglutinated sperm to dilutions of 2^{-8} to 2^{-10} . No sera were pooled.

Tests, assays and absorptions. The sperm agglutination tests were performed by mixing one volume (usually one drop) of 1% sperm suspension with two volumes (2 drops or 0.1 ml.) of serum in depression slides. The salt concentration of sera was adjusted to that of sea water by dialysis or addition of an equal volume of $1.73 \times$ sea water. Reactions were recorded after the mixtures had remained for three hours at room temperature. Parallel tests with control (pre-injection) serum were performed in all experiments to control for nonspecific reactions.

The agglutination of *Arbacia* sperm with antiserum gave a regular pattern. When diluted serially in sea water the antiserum gave titers with sharp, reproducible end points. However, the morphology of the agglutinates varies upon dilution of the antiserum. Antiserum at dilutions to 2^{-5} yielded net-like agglutinates involving head-to-head, head-to-tail and tail-to-tail agglutination. Agglutination at higher antiserum dilutions was head-to-head, the sperm was motile and the agglutinates were "bouquet-like" in structure. Sperm in such dilute antisera fertilized eggs and became more active on addition of fertilizin. This last effect was associated with a breakdown of the antiserum agglutinates.

The absorptions were done with varying amounts of material. Absorbing antigen and antibody were usually allowed to react for three hours at room temperature. Absorption over a longer time did not result in lower titers.

Isolation of sperm tails and heads. Isolated sperm tails and heads were used in absorption and agglutination experiments. In order to separate sperm tails and heads washed, living or formalin-killed sperm (25% suspension) was subjected to the shaking action of the Mickle disintegrator for 20 minutes at room temperature. The resulting material was then centrifuged at low speed at 4° C. to sediment the sperm heads. Suspensions of tails and of heads were washed when necessary by high and low speed centrifugation, respectively, at 4° C.

RESULTS

In this investigation primary interest centers about the sperm "surface" as opposed to "subsurface" or interior material with antigenic properties. Although the sperm surface may be difficult to define or delimit at the molecular level, present requirements are satisfied by an operational definition. According to this definition, sperm surface antigens (specific combining sites) are those antigens so situated that their combination with antibody under appropriate conditions results in sperm agglutination. In any event information concerning the role of the sperm surface in fertilization is the ultimate objective here and antigenic groups that are available for participation in the agglutination reaction should be sufficiently accessible for interaction with the egg surface in the initial stages of fertilization.

In the experiments described below some attention is given to the antigenic composition of sperm extracts. It is clear that the "surface" vs. "subsurface" origin of soluble antigens in sperm extracts is not readily established. For example, a single antigenic group might be readily extracted from the cell "interior" and at the same time exist in bound, unextractable and insoluble form at the cell "surface." However, removal of sperm agglutinating antibodies from antisera by absorption with sperm extracts serves to identify and separate antibodies against sperm surface antigens irrespective of the origin of the absorbing antigens.

Such absorbed sera in turn can serve to separate and identify sperm surface antigens. The experiments described below should be considered in this conceptual framework.

A) Identification of sperm surface antigens by interspecific reactions

In order to identify sperm surface antigens, cross-absorption tests were performed in interspecific, agglutinating combinations of sperm and antisera. Six foreign sperms were tested for cross-reaction against antisera prepared against sperm of four of the species. With the exception of the combination *Mellita* sperm

TABLE I
Sperm agglutinating action of antisera before and after absorption with sperm of various species

Sera		Immune sera Tested with					
Prepared against sperm	Abs. with sperm	A*	L	E	M	P	Asterias
A*	—	+	+	+	0	+	0
A	A	0	0				
A	L	+	0	0			
A	E	+	+	0			
A	E + L	+	0				
L	—	+	+	+	+(weak)	+	+(weak)
L	L	0	0				
L	A	0	+	0			
L	E	+	+	0			
L	E + A	0	+	0			
E	—	+	+	+	+		+
E	L	0	0	+			
E	A	0	+				
E	A + L	0	0	?			
Asterias	—	0	0		0		+

*A = *Arbacia*; E = *Echinarachnius*; L = *Lytechinus*; M = *Mellita*; P = *Plagiobrissus*.
Control (pre-injection) sera failed to agglutinate sperm in parallel tests to those in the table.

vs. *Arbacia* antiserum, cross-reactions were found among all echinoid combinations tested. These relations are summarized in Table I.

Certain of these cross-reacting combinations were used in cross-absorption experiments. These included the series *Arbacia*, *Lytechinus* and *Echinarachnius* as seen in Table I. Absorption with the homologous sperm rendered antisera non-agglutinating for the cross-reacting heterologous species. In every case complete absorption with an heterologous sperm yielded a serum which still agglutinated the homologous species. Furthermore, in most cases such absorbed sera agglutinated the other heterologous species as well. Thus anti-*Arbacia* serum absorbed with *Echinarachnius* sperm still agglutinated *Lytechinus* and *Arbacia* sperm.

Evidently, then, *Lytechinus* and *Arbacia* sperm possess surface antigens lacking on *Echinarachnius*. A further absorption of the *Echinarachnius* sperm absorbed anti-*Arbacia* serum with *Lytechinus* sperm yielded a serum which failed to agglutinate *Lytechinus* but still agglutinated *Arbacia* sperm. Accordingly, it is concluded that the *Arbacia* sperm surface possesses at least three antigens or antigenic complexes, one specific for *Arbacia*, a second shared with *Lytechinus* and a third shared with *Echinarachnius*. It is of some interest that *Lytechinus* sperm absorbs all *Echinarachnius* sperm agglutinins from anti-*Arbacia* sperm serum. This indicates that all the "*Arbacia* antigens" present on the *Echinarachnius* sperm surface are also present on the *Lytechinus* sperm. Comparable results were obtained in absorption experiments using anti-*Lytechinus* sperm serum (Table I).

B) Sperm surface antigens in sperm extracts

In the previous section the *Arbacia* sperm surface was shown to possess at least three antigens or antigenic complexes. In view of the possible role of insoluble as well as soluble sperm surface substances in fertilization (see Metz, 1957a), it seemed of interest to examine sperm extracts for soluble antigens in common with sperm surface antigens. This matter is of special interest because it concerns the question whether the antifertilizin extracts from sperm contain the full complement of sperm surface antigens. In one series of experiments soluble antigen extracts were prepared by freeze-thawing *Arbacia* sperm. This is one method for preparing sperm antifertilizin (Tyler, 1939). All extracts were subject to low speed centrifugation ($3000 \times g$ for 20 minutes) to remove fragments of the sperm surface. Certain preparations were also subjected to high speed centrifugation ($26,000 \times g$) to remove the larger sub-cellular particulates. Constant amounts of anti-*Arbacia* sperm serum were then absorbed with increasing amounts of the sperm extracts. The absorbed sera were then assayed for sperm agglutinating action. As seen in Figures 1a, 3a, 4a, and 6a, increasing amounts of sperm extract lowered the sperm agglutinin titer until a constant value was reached. Beyond this point increasing amounts of extract fail to affect the sperm agglutinating action. The extracts failed to remove all of the sperm agglutinins (seven experiments). Accordingly, it appears that the extracts do not contain all of the sperm surface antigens. Some of these evidently are not extractible in sea water by freeze-thawing and do not appear as components in extracted "antifertilizin." Most of these antigens are heat-labile. If the frozen-thawed extract is heated a precipitate begins to form at 55°C . and after further heating for one minute at 100°C . the supernatant has reduced antifertilizin activity and lowers the sperm agglutinating titer of antiserum only one dilution step (compare Figure 1, a and b).

In view of the antibody absorbing action of extracts prepared by freeze-thawing sperm, it seemed of interest to examine extracts prepared by other methods for antigens in common with those present on the intact sperm surface. Accordingly, extracts were prepared by heating (100°C . for one minute) followed by centrifugation, by disruption in the Mickle disintegrator, and by acid extraction.

As seen in Figure 2a (typical of two experiments), extracts prepared by heating had little if any effect on the sperm agglutinating action of anti-sperm serum. Evidently, then, heat extraction yields few if any sperm surface antigens. However, antigenic material is present in extracts prepared by heating, for such extracts

produce one precipitin band in agar when diffused against antiserum (to be published). Possibly this is sub-surface material. If so, it suggests that the antifertilizin (egg jelly precipitating and fertilizin neutralizing) activity of extracts prepared by heating (Frank, 1939) is not due to sperm surface material. Finally, as seen above, frozen-thawed extracts contain insignificant amounts of surface antigenic material after heating to 100°C . Extracts prepared by action of the Mickle disintegrator also neutralized the sperm agglutinating action of anti-whole sperm serum. As seen in Figure 2c (two experiments), such absorption reduced the agglutinin titer to a constant level, but failed to remove all agglutinins.

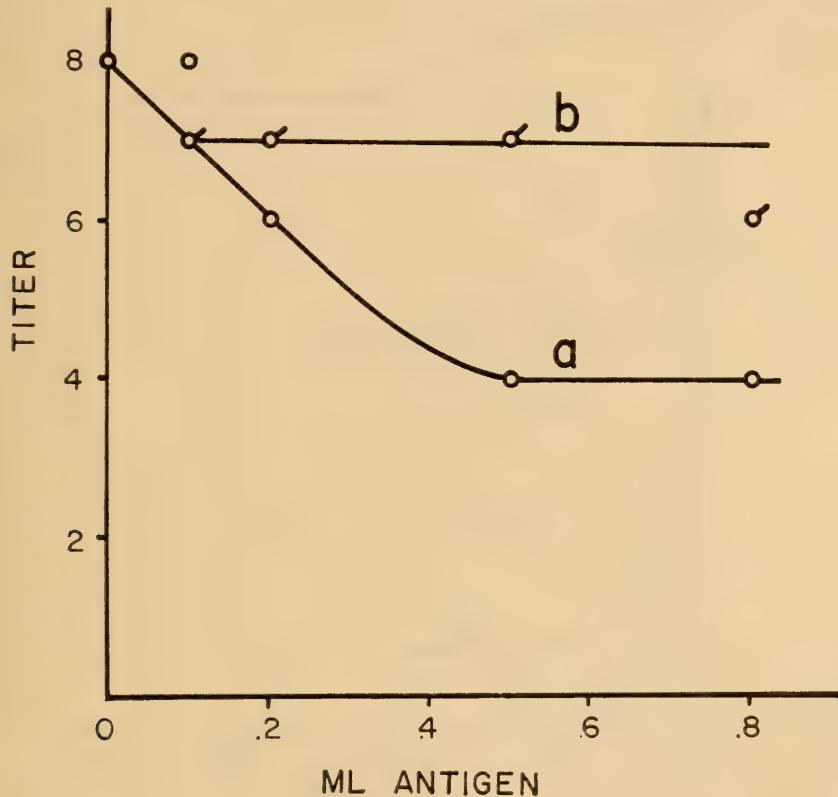


FIGURE 1. Effect of absorption with increasing amounts of antigen on sperm agglutinating action of anti-*Arbacia* sperm serum. Curve (a): Antiserum absorbed with frozen-thawed sperm extract, tested on *Arbacia* sperm. Curve (b): Antiserum absorbed with heated (100°C , one minute) frozen-thawed sperm extract, tested on *Arbacia* sperm. Ordinate: Titer = $-\log_2$ of highest antiserum dilution giving sperm agglutination. Abscissa: ml. antigen added to 0.5 ml. antiserum.

Acid extraction of sperm also yields antifertilizin (Tyler and O'Melveny, 1941). In one experiment anti-whole sperm serum was absorbed with increasing amounts of neutralized pH 3.0 sperm extract. Figure 2b again shows reduction of the agglutination titer to a constant level. In an additional comparative experiment sperm suspensions (12.5%) were extracted at pH 3.2, 7.2 and 9.0 for six hours. The extracts were subsequently tested for antibody absorbing action. The pH 7.2 ("aged sperm") extract lowered the sperm agglutination titer a maximum of one-half (one-fold) whereas the acid and alkaline supernatants lowered the titer of the antiserum a maximum of $\frac{1}{8}$ (three-fold). Thus, acid and alkaline extracts

of sperm, like frozen-thawed and Mickle extracts, contain some but not all of the antigenic groups present on the sperm surface.

Relation of antigens in sperm extracts to fertilizin. In view of the fact that the soluble antigen preparations contain antifertilizin, it seemed of interest to examine the relations of such extracts and of anti-sperm serum to fertilizin. Specifically, it appeared of interest to test for action of fertilizin on the antiserum neutralizing action of soluble sperm antigen extracts (antifertilizin). To test for neutralizing

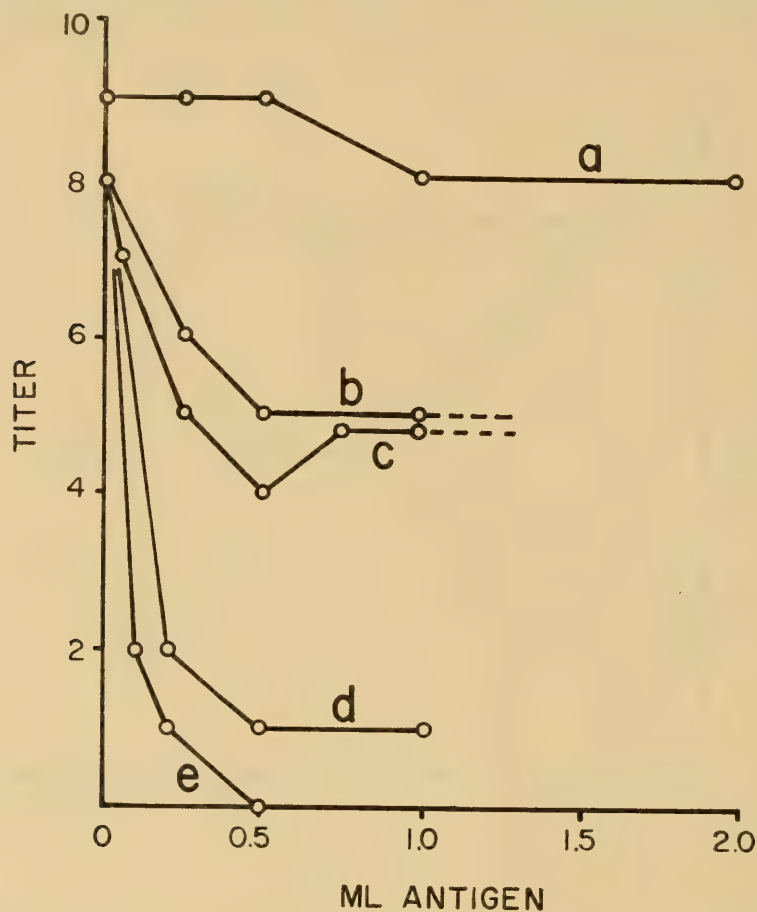


FIGURE 2. Effect of absorption with increasing amounts of antigen on sperm agglutinating action of anti-*Arbacia* sperm serum. Curve (a): Absorbing antigen prepared by heating (100°C ., one minute) sperm. Curve (b): Absorbing antigen prepared by extracting sperm in pH 3.0 sea water. Curve (c): Absorbing antigen prepared by Mickle disintegration of sperm. Curve (d): Absorbing antigen: isolated sperm heads. Curve (e): Absorbing antigen: whole intact sperm. Absorbing antigen for curves (a), (b) and (c) centrifuged $3000\times g$. Each curve represents a different experiment. Ordinate and abscissa as in Figure 1.

action, fertilizin and antiserum were mixed in varying proportions and the sperm agglutinin titers determined. The experiment is complicated by the fact that fertilizin and antiserum both agglutinate sperm. To avoid confusion from the action of the two different agglutinins, the sperm was examined for agglutination after sufficient time (three hours) to permit reversal of any agglutinating action of fertilizin (see methods section). As seen in Figure 3b the sperm agglutinin titer of the antiserum was not reduced by fertilizin. It appears, then, that the

sperm surface and fertilizin do not have antigenic combining sites in common which are essential for sperm agglutination.

On the other hand, the combining sites of the soluble antigens in frozen-thawed extracts evidently are blocked by fertilizin. This is again seen in Figure 3, curve c. In this experiment decreasing amounts of fertilizin were mixed with increasing amounts of the sperm surface extract (antifertilizin). The mixtures were subsequently used to absorb the anti-sperm serum. When the extract-to-fertilizin ratio equalled 0.143 (0.25 ml. extract) the mixture failed to affect the

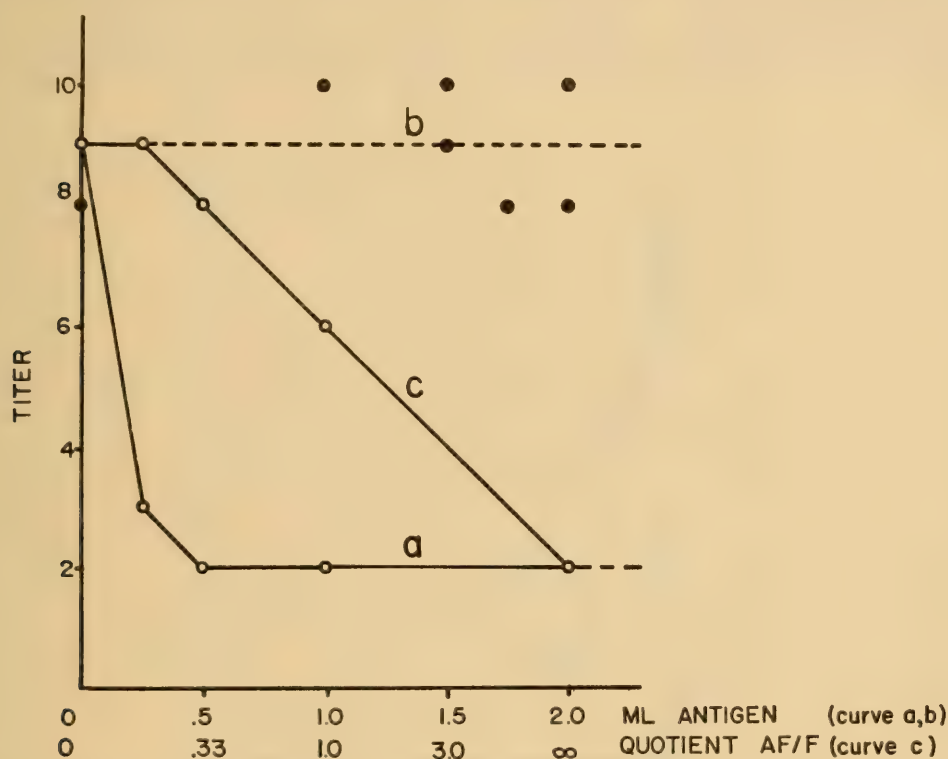


FIGURE 3. Effect of absorption with increasing amounts of antigen on the sperm agglutinating action of anti-*Arbacia* sperm serum. Curve (a): Serum absorbed with sperm extract (antifertilizin) prepared by freeze-thawing sperm and centrifuging at $3000 \times g$ for 20 minutes. Curve (b): Antiserum absorbed with fertilizin. Curve (c): Antiserum absorbed with antifertilizin-fertilizin mixtures. Ordinate: Titer = $-\log_2$ of highest antiserum dilution giving sperm agglutination. Abscissa for (a) and (b): ml. antigen added to 0.5 ml. antiserum. For (c): Quotient antifertilizin/fertilizin/0.5 ml. antiserum. Two ml. fertilizin and no antifertilizin at quotient = 0. The same fertilizin preparation was used for curve b and c. Absolute amounts of antifertilizin, antisera and total volumes in curves (a) and (c) are identical along the abscissa.

sperm agglutinin titer of the anti-sperm serum, whereas the same amount of antifertilizin alone reduced the agglutinin titer six-fold. Increase in the proportion of antifertilizin resulted in mixtures with antiserum neutralizing action. This result shows that fertilizin neutralizes the ability of the frozen-thawed extract to absorb sperm agglutinins from the anti-sperm sera.

Distribution of "soluble" surface antigens. Data in the previous section show that extraction of sperm yields solutions of soluble sperm antigens and that these neutralize some of the antibodies (agglutinins) for the sperm surface. On the

other hand the extracts fail to neutralize all of the sperm agglutinating antibodies. Accordingly, the extraction procedures fail to remove some "insoluble" antigenic material from the sperm. Although the antigens in the preparation may not be extracted from the sperm surface they are antigenically related to, and therefore serve to identify, surface antigens. In an attempt to localize these "soluble antigens" on the sperm surface, antisera were treated with extracts prepared by freeze-thawing (antifertilizin) and then tested for agglutinating action on suspensions of isolated sperm heads, tails and intact sperm. As seen in Figure 4 such extracts completely neutralized the agglutinins for sperm tails. Evidently, then, the sperm tail surface possesses only soluble antigen. In this connection it is of interest that agar diffusion precipitin tests show only a single band when frozen-thawed extract of isolated

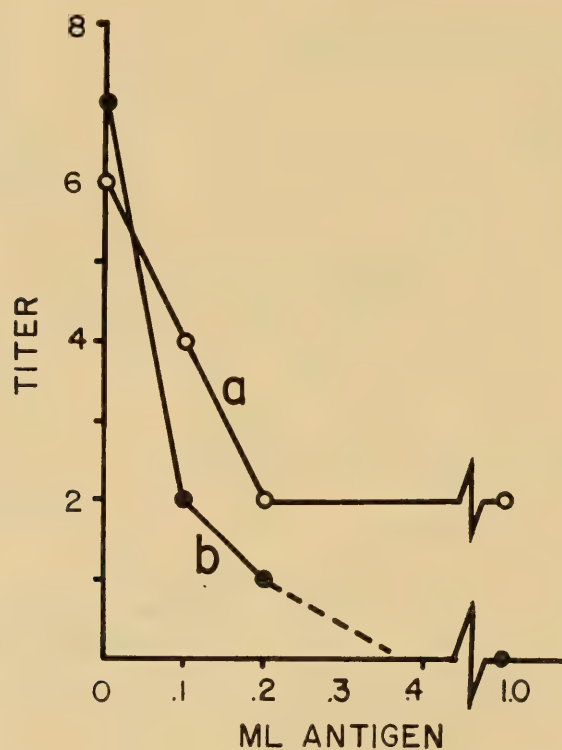


FIGURE 4. Agglutination of whole sperm and isolated sperm tails with anti-*Arbacia* whole sperm serum absorbed with frozen-thawed whole sperm extract (antifertilizin). Curve (a): Agglutination of whole sperm. Curve (b): Agglutination of isolated tails. Ordinate and abscissa as in Figure 1.

sperm tails is diffused against anti-whole sperm serum. This single band merges with one of four produced by whole sperm extract (to be published). These observations show that the insoluble antigens are confined to the sperm head. This view is confirmed by examination for agglutination of whole sperm and heads by the absorbed sera. As seen previously (Fig. 3a) the agglutinin titer for whole sperm is reduced to a constant level by absorption with frozen-thawed extracts. On the other hand, such extracts had no apparent effect on the ability of anti-sperm serum to agglutinate isolated sperm heads. Evidently, the surfaces of the isolated sperm heads and the frozen-thawed extracts do not have antigens in common and the sperm heads, prepared by treatment in the Mickle disintegrator, lack (have lost?) soluble surface antigenic material.

Regional localization of sperm surface antigens. In a previous section it was shown that whole sperm extract absorbed the agglutinins for isolated *Arbacia* sperm tails but failed to absorb all agglutinins for isolated sperm heads. This result indicates that the sperm tail antigens are soluble, whereas some or all of the antigens on the sperm head surface are insoluble. It seemed of interest to extend this study to a more direct analysis of differences between sperm heads and tails. To achieve this the method used by Henle *et al.* (1938) for bull sperm was employed. *Arbacia* sperm heads and tails were isolated by treatment in the Mickle disintegrator fol-

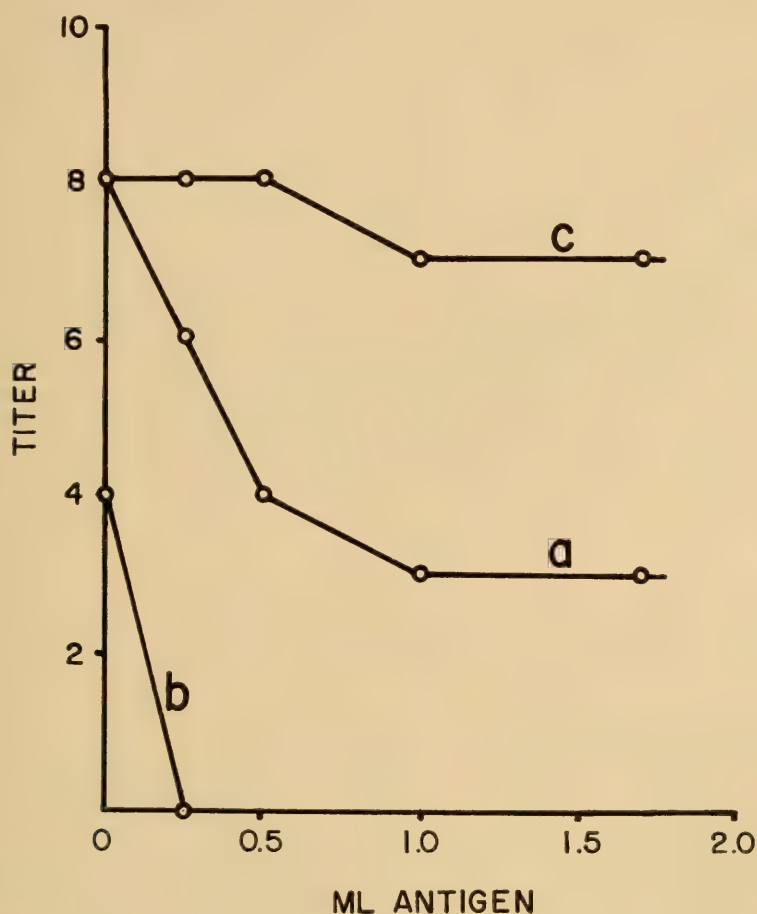


FIGURE 5. Sperm agglutinating action of anti-*Arbacia* whole sperm serum after absorption with sperm tails isolated from formalin-killed sperm. Curve (a): Tested on living *Arbacia* sperm. Curve (b): Tested on sperm tails isolated from formalin-killed sperm. Curve (c): Tested on sperm heads isolated from formalin-killed sperm. Ordinate and abscissa as in Figure 1.

lowed by differential centrifugation. The suspensions of isolated heads and tails were then used to absorb anti-whole sperm serum. Preliminary experiments showed that isolated tails always reduce the sperm agglutination titer in absorption experiments. Isolated sperm heads, however, showed varying results and require further study. The inconsistencies may be related to the presence of very readily soluble sperm surface antigens which appear in washings of the sperm.

Unfortunately, treatment of living sperm in the Mickle disintegrator resulted in considerable fragmentation of the sperm tails. This was especially true in the

preparation of large amounts of tails for absorption experiments. To facilitate isolation of unbroken tails sperm were first fixed in 1% formalin, washed in saline and the tails shaken from the remainder of the sperm in the Mickle disintegrator.

In using formalin-fixed sperm it is assumed that the formalin treatment does not alter the antigenic structure of the sperm surface. This assumption is valid to the extent that both living and formalin-killed whole sperm and sperm fragments agglutinated to the same titer with anti-sperm serum and that living and formalin-fixed material showed parallel absorbing action in preliminary experiments. For

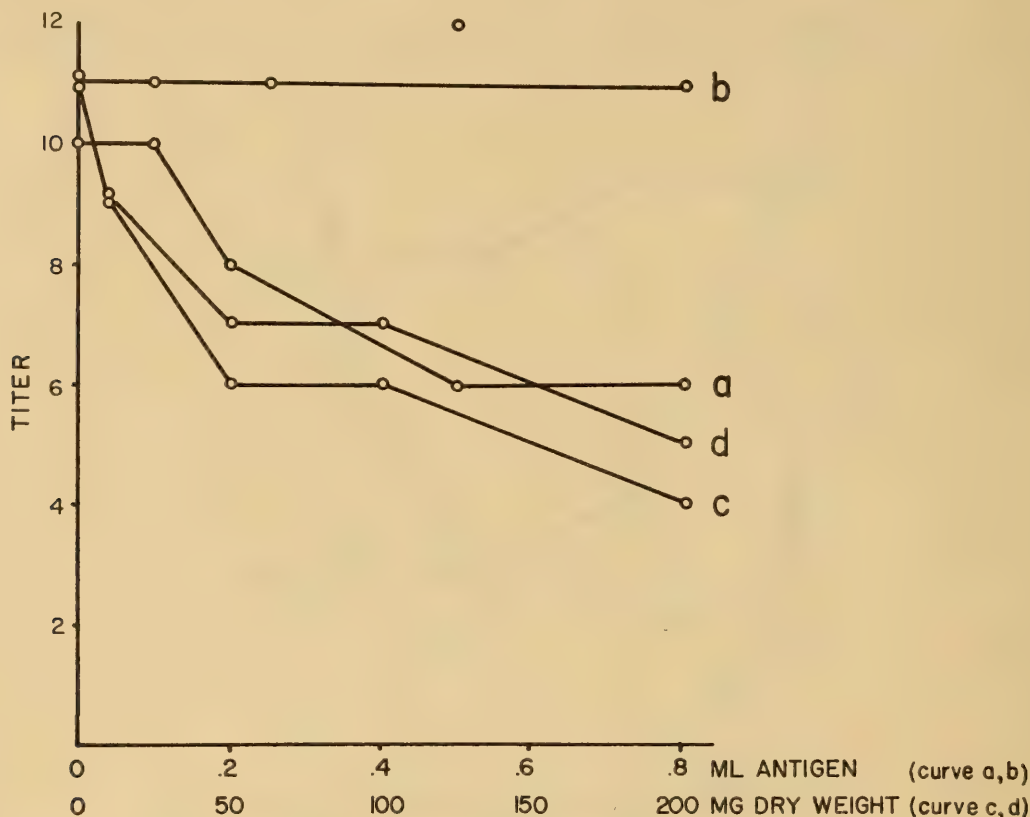


FIGURE 6. Sperm agglutinating action of anti-*Arbacia* whole sperm serum absorbed with *Arbacia* antifertilizin and sperm "ghosts." Curve (a) and (b): Absorbing antigen, frozen-thawed sperm extract. (a): Tested on *Arbacia* sperm. (b): Tested on *Lytechinus* sperm. Abscissa: ml. antigen added to 0.2 ml. antiserum. Curve (c) and (d): Absorbing antigen, frozen-dried "ghosts." (c): Tested on *Arbacia* sperm. (d): Tested on *Lytechinus* sperm. Abscissa: mg. dry weight. Ordinates for (a), (b), (c) and (d): Titer = $-\log_2$ of highest antiserum dilution giving sperm agglutination.

a final experiment washed sperm was fixed in formalin (1%), washed and treated for 30 minutes in the Mickle disintegrator. The tails were then isolated from the intact sperm and heads by centrifugation. Finally, the isolated tails were used in varying amounts to absorb the anti-whole sperm serum. This absorbed serum failed to agglutinate tails (Fig. 5b). However, it did agglutinate isolated sperm heads (Fig. 5c) and living whole sperm (Fig. 5a), but in both instances the absorbed serum showed lower titers than the unabsorbed serum. Evidently, then, the sperm heads possess a head specific antigen or antigens which are not present on sperm tails. The reduction in titer of sperm head agglutination by absorption

with tails indicates that some tail antigenic material is also present of the sperm heads.

Analysis of insoluble sperm surface antigens. In order to confirm the presence of water-insoluble sperm surface antigens it seemed of interest to attempt to absorb sera with sperm from which the soluble sperm antigens had been removed. Sperm "ghosts" were prepared by freezing 25% sperm, removing the supernatant, re-freezing the re-suspended residue and finally washing the insoluble material five times in sea water. As seen in Figure 6a, the initial extract of soluble antigens (antifertilizin) lowered the sperm agglutinin titer four-fold whereas the extracted "ghosts" when used in excess lowered the titer seven-fold (Fig. 6c). Evidently, then, the extracted "ghosts" possess insoluble antigens that are not present in the soluble fraction.

Furthermore, it is of interest that these *Arbacia* "ghosts" also lowered the sperm agglutinin titer for *Lytechinus* sperm (curve 6d), whereas the absorption with frozen-thawed extract (Fig. 6b) did not affect the titer for *Lytechinus*. It remains for future experiments to determine if all the antigens common to the two species are insoluble, and if the ones in the extracts are the specific antigens.

Presence of sperm surface components in body fluid and egg extract. Results from several experiments show that neither body fluid from male or female sea urchins nor egg extract contains sperm surface components involved in antiserum agglutination. Absorption of whole sperm antiserum with these extracts did not lower, or lowered only one step, the titer for sperm agglutination.

DISCUSSION

In view of the striking morphology of the spermatozoan, the multiple antigen structure of cell surface generally (*e.g.* erythrocytes) and the presence of at least three distinct surface antigens on bull sperm (Henle *et al.*, 1938), and two on *Strongylocentrotus* sperm (Tyler, 1949), it is not surprising to find several antigens on the *Arbacia* sperm surface. The distinct head and tail antigens found on *Arbacia* sperm conform to the pattern found for bull sperm. It seems likely that tests of a wider range of heterologous species would reveal more cross-agglutination reactions between foreign sperm (*e.g.* Tyler, 1949) and the anti-*Arbacia* sperm serum. Absorption of the antiserum with such foreign sperm might reveal more antigens in addition to the three so far demonstrated on the *Arbacia* sperm surface. Furthermore, a more detailed analysis with such absorbed sera, such as testing for agglutinating action on isolated sperm heads and tails, should localize the individual antigens or antigenic complexes.

The number and distribution of the sperm surface antigens is not without interest, for this information contributes to the knowledge of sperm surface architecture. Furthermore, such antigens could be used as additional points of attack in studies on the role of the sperm surface in fertilization. However, the relationship of the sperm surface antigens to antigens present in sperm extracts appears to be of more immediate interest.

Extracts of sea urchin sperm prepared by heating (Frank, 1939), freeze-thawing (Tyler, 1939) and acid extraction (Tyler and O'Melveny, 1941) contain an agent or agents called antifertilizin, which neutralizes the sperm agglutinating action of fertilizin, agglutinates eggs and precipitates the egg jelly. This antifertilizin in

sperm extracts is assumed to come from the sperm surface and to include the specific sperm surface combining sites with which fertilizin reacts when it reversibly agglutinates sperm.

In support of this view Tyler (1949) extracted antifertilizin from the *Strongylocentrotus* sperm by acid treatment. Subsequent examination of the cells with the electron microscope revealed a partial breakdown of the sperm head surface, suggesting this as the site of origin of the antifertilizin. However, the situation is probably more complicated than this, for as seen in the present study absorption of anti-whole sperm serum with sperm extracts (antifertilizin) removes tail agglutinins but has little effect on the head agglutinins. This suggests that the antifertilizin may be present in large amounts on the sperm tail.

Furthermore, agar gel precipitin tests show that the antifertilizin extracts prepared by freeze-thawing regularly contain three to four antigens (to be published). The surface vs. sub-surface origin of these antigens is not immediately clear. However, the fact that this mixture of antigens will remove some sperm agglutinins from anti-sperm serum suggests that at least one of the antigens is of sperm surface origin. But even this experiment does not exclude the possibility that the absorbing antigen(s) possesses a combining group in common with the sperm surface but is actually extracted from the interior of the sperm.

In further attempts to identify these antigens the antigen neutralizing action of fertilizin was examined. Following treatment with fertilizin, antifertilizin failed to lower the sperm agglutination titer of the antiserum. Clearly, then, fertilizin can neutralize all the sperm surface antigens in the frozen-thawed extract. The fertilizin may be highly specific in action and neutralize only one of the four antigens. If this is the case, it follows that the extract prepared by freeze-thawing contains only one antigen in common with the sperm surface. In any event, the antigen or antigens involved appear to be related mainly to the antigens of the sperm tail (see above).

Studies on the role of specific egg and sperm substances in metazoan fertilization have been concerned mainly with soluble agents. It is of some interest, then, to find that extracts of sperm do not contain all of the surface antigens. Evidently, some antigens are either destroyed by the extraction procedure or remain in the insoluble residue. The second alternative seems the more likely in view of the nature of the extraction procedure. Indeed the absorption experiments with sperm "ghosts" show that insoluble antigens survive the extraction procedure. The role in fertilization of such insoluble sperm surface antigens remains to be investigated. The specific combining groups on the sperm surface that function in the antiserum agglutination reaction may not be identical with molecular configurations that function in fertilization. Nevertheless it seems likely that such material may form a part of, or be spatially related to, molecular surface patterns that do function in the union of sperm and egg. This apparently is the case in *Paramecium* (Metz, 1954).

Consideration here has been given only to the sperm head and tail. A more detailed study should take into account the subdivisions of these structures, such as the axial tip of the tail, and the midpiece and acrosome of the head. Furthermore, especial attention should be given to comparison of the antigens of the sperm before and after acrosomal filament discharge, for at least in some forms (*e.g.* starfish) activation of the egg results from interaction of the egg surface with the tip

of the acrosomal filament (see Metz, 1957b; Colwin and Colwin, 1957; Collier, 1959; Dan, 1956).

SUMMARY

1. Sperm surface antigens are defined as those antigens which are detected by sperm agglutinating action of antisera.
2. Interspecific sperm agglutination tests show one or more sperm surface antigens common to all of the five echinoids tested except for a single combination.
3. The *Arbacia* sperm surface possesses a minimum of three antigens or antigenic complexes in common with other species. One of these is shared with *Lytechinus* sperm, a second with both *Lytechinus* and *Echinarachnius* and a third is not present on sperm of these species.
4. *Arbacia* sperm extracts prepared by freeze-thawing, Mickle disintegration or pH 3 treatment all contain "soluble" antigens in common with the sperm surface. The *Arbacia* sperm surface also possesses "insoluble" antigens which do not appear in the above extracts.
5. The "soluble sperm surface" antigen(s) in extracts is neutralized by fertilizin from *Arbacia* eggs.
6. It is present on the sperm tail surface and possibly to some extent on the sperm head.
7. The insoluble sperm surface antigen(s) is confined to the sperm head.

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THE RELEASE OF EFFERENT NERVE ACTIVITY IN THE ROACH, PERIPLANETA AMERICANA, BY EXTRACTS OF THE CORPUS CARDIACUM¹

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Several attempts have been made to correlate patterns of endogenous activity as observed in isolated portions of invertebrate nervous systems with the normal behavior of the intact animal (von Holst, 1934; Roeder, 1955; and Harker, 1956 and 1958). Roeder, Tozian and Weiant (1960) found that decapitation of roaches or of mantids markedly increases the level of endogenous activity in the efferent nerves from the cercal ganglion which innervate the phallic musculature and in the efferent nerves from the metathoracic ganglion which innervate the thoracic musculature. As the activity in the phallic nerves increases, rhythmic bursts of nerve spikes are seen. These motor bursts on the several nerve branches leading to the phallomeres are not obviously coordinated, but they result in ordered, rhythmic movements of the genitalia. The behavioral significance of this endogenous nerve activity is suggested by the observation that in male mantids the copulating reflexes appear with great intensity following decapitation. Indeed, under natural conditions, the male is often beheaded by the female before he mates with her (Roeder, 1935). Control of this sexual behavior and of the efferent nerve activity in the nerves to the phallomeres and to the thoracic musculature appears to be effected through the agency of an inhibiting center in the subesophageal ganglion.

In most of these experiments (Roeder, Tozian and Weiant, 1960) there was a time lag of five to fifteen minutes between the removal of the head and the appearance of bursts of spikes in the efferent nerves. This time lag might result from a decreasing discharge of severed inhibitor neurones, augmented by injury potentials. On the other hand, it might represent the time necessary for the inactivation or removal of an inhibiting neurohormone. Such a material, if present, could be transported by proximo-distal flow down axons within the nerve cord to the site of its action.

A variety of substances are to be found in roaches which affect their behavior and some of these may be produced within the central nervous system itself (Beament, 1958; Colhoun, 1958, 1959; Smyth, 1959; and Sternberg and Kearns, 1952). The work of Özbas and Hodgson (1958) showed that the injection of corpus cardiacum extracts produced stereotyped behavior in roaches and that these extracts had a pronounced inhibitory effect on the activity in isolated roach abdominal nerve cords. Their results led us to attempt to block the inhibitory influences

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impinging on the efferent nerve cells in the cercal and metathoracic ganglia by the use of similar extracts.

MATERIALS AND METHODS

Most of the observations of activity in efferent nerves to the phallomeres and in the connectives of the abdominal nerve cord were made using male *Periplaneta americana* as test animals. For these experiments the animals' wings and legs were removed and the bodies were pinned dorsal side down on a cork. Most of the abdominal contents were removed. Nerve IX or nerve X from the cercal ganglion (Fig. 1, E²) was picked up on a small tapered silver wire hook electrode and allowed partially to dry. In many experiments afferent impulses in the nerve and the muscle potentials resulting from the efferent activity were eliminated by cutting the nerve distal to the recording electrode. The indifferent electrode was a chlorided silver wire touching the inner abdominal wall. Simultaneous recordings of nerve activity in the connectives of the abdominal cord were made by raising a connective on a pair of silver hook electrodes (Fig. 1, E¹). The input from the phallic nerve fibers was amplified with a Grass P6 preamplifier and observed on a Dumont 304A oscilloscope. The amplified signals were also recorded on one channel of a Grass Polygraph inkwriter using a 5P3 integrator preamplifier. The activity from the abdominal connectives was amplified by a 5P3 preamplifier and appeared on the other channel of the inkwriter. The two inputs could be transposed at any time so that visual observations or photographs could be made of either the nerve impulses from the cord or of those from the phallic efferent nerves. Meanwhile, a continuous record of the integrated activity from both sources appeared on the inkwriter.

Endogenous efferent nerve activity in the thoracic region was sampled from an electrode pair placed under nerve 3 (Fig. 1, E³) from the left side of the metathoracic ganglion. The method has been described previously (Weiant, 1958). All other nerves arising from the thoracic ganglia were severed, eliminating all sensory input from these segments. The longitudinal connectives between the thoracic ganglia were left intact, as were the connections of the thoracic cord with the head ganglia and abdominal ganglia. Peripheral connections of extra-thoracic regions of the nervous system were also left intact. Activity of the internuncial fibers within the thoracic connectives was monitored simultaneously with that of nerve 3 by placing a pair of electrodes under the thoracic connectives above and below the prothoracic ganglion (Fig. 1, E⁴).

Many of the same extract samples were tested for their action both on the phallic motor nerves and on the thoracic efferent fibers. In experiments with both of these preparations, injections into the head capsule of 0.01 to 0.02 ml. of extract were made as close to the subesophageal ganglion as possible. In some tests with the thoracic preparation a drop of extract was applied directly on the metathoracic ganglion; in other experiments using the abdominal preparation, several drops of extract were used to bathe the last three abdominal ganglia with their connectives. Some tests were conducted on *Periplaneta* from which the corpora cardiaca had previously been removed.

Extracts were made from the corpora cardiaca of the following species of roaches: *Blaberus craniifer*, *Blaberus giganteus*, *Periplaneta americana*, *Leucophaea*

maderae, *Byrsotria fumigata*, and *Diploptera punctata*. Extracts were from the glands of adult animals except in one case in which an extract was made from the corpora cardiaca of penultimate stage nymphs of *Periplaneta*.

The paired corpora cardiaca were excised according to the technique of Özbas and Hodgson (1958) and placed on the end of a small glass pestle. The glands were removed as quickly as possible after the donor roaches had been caught in order to minimize the loss of active material due to excitation of the roach (Hodgson, personal communication). When the requisite number of corpora cardiaca had been collected, they were triturated in a small mortar. Either Hoyle's or Pringle's insect saline was added from a hypodermic syringe to dilute the extract to the concentration desired. Pringle's proved to be the more satisfactory saline for maintaining the nerve activity in the preparations.

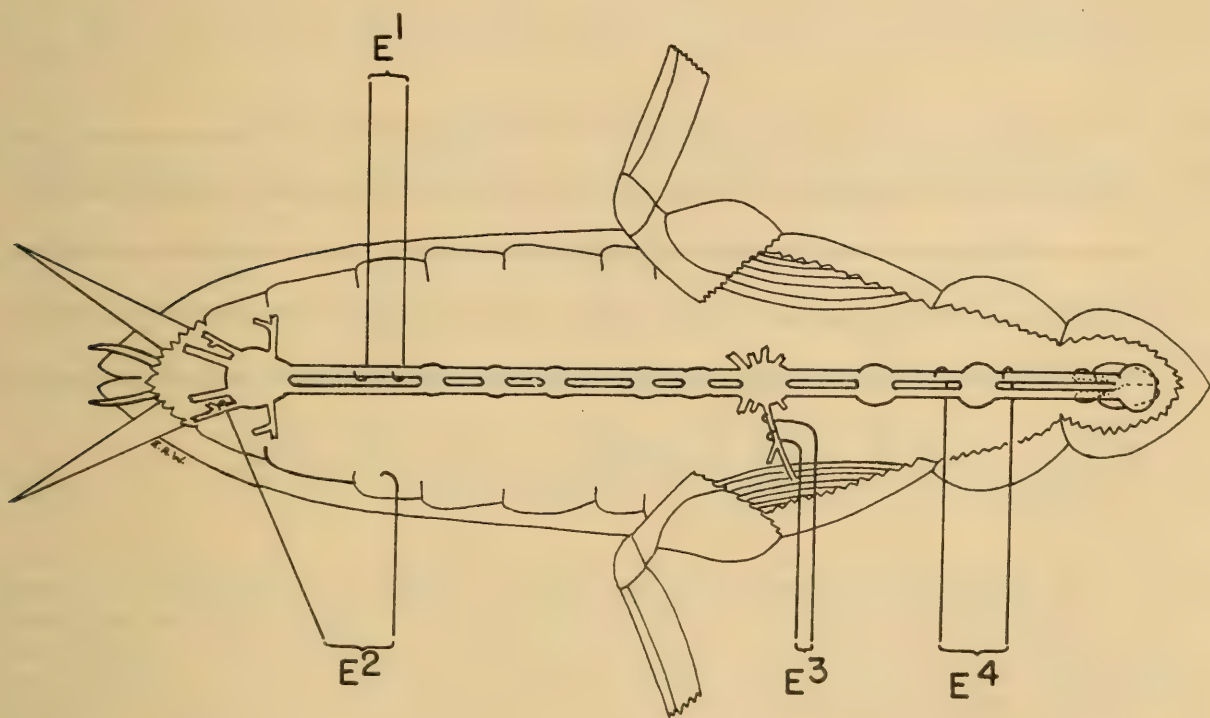


FIGURE 1. Diagram of the central nervous system of *Periplaneta americana* showing placement of electrodes.

In many of the experiments the mortar containing the extract was heated at 95° to 100° C. for five minutes. A coagulum formed in the bottom of the mortar, leaving a fairly clear solution which was used for the tests. Longer exposures to heat seemed to impair the activity of the extract, but a brief period of heating left the potency unchanged. Unheated extracts lost their activity somewhat after four to five hours at room temperature or after about ten hours at 12° to 14° C. Heating approximately doubled the time that the extracts would remain active.

Four experiments were conducted with extracts which were quick-frozen on blocks of dry ice and kept frozen for periods of twenty-four hours to a week. Two of these extracts were heated before being frozen. None of the quick-frozen extracts showed any loss of activity. This seems a promising method for preserving such material.

RESULTS

Efferent nerve activity of the last abdominal ganglion. The effects of decapitation or of nerve cord transection on efferent fiber activity of the last abdominal ganglion have been described in detail elsewhere (Roeder, Tozian and Weiant, 1960). In a roach with the nervous system intact, spike potentials are observed in only a few efferent fibers of the nerves supplying the phallic musculature. In those fibers which are active, spikes recur regularly and at a relatively low frequency. If the animal is decapitated, there is a brief injury discharge followed by a return to the previous level of activity. After five to ten minutes the number of active fibers in the phallic nerve begins to increase, and a slowly-rising crescendo of activity is observed due to a steady increase in the spike frequency in both newly—and previously—active fibers. Typically this increased efferent discharge gradually becomes patterned into a sequence of impulse bursts. At first the bursts are isolated, but they soon become rhythmic and increase steadily in frequency for the next

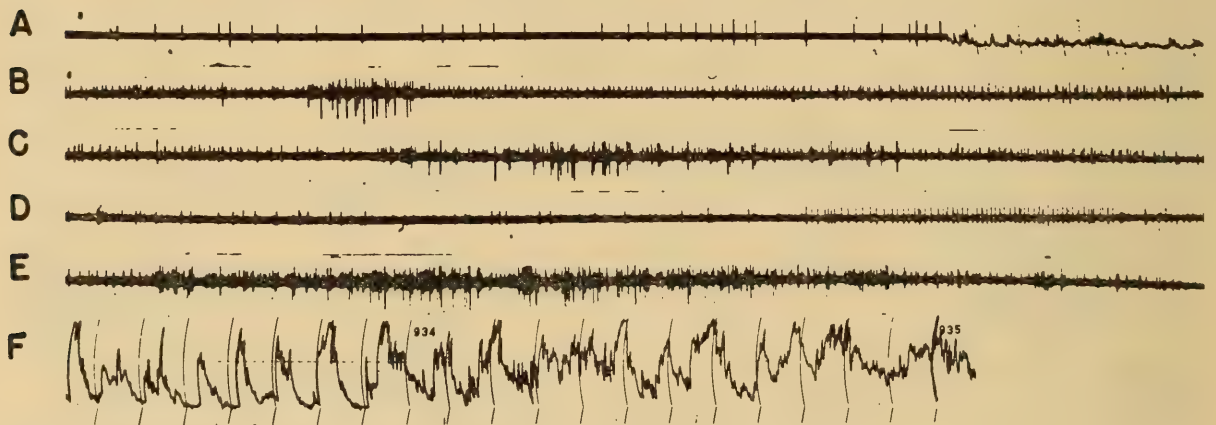


FIGURE 2. A. Oscilloscope record of impulses on the phallic nerve of a normal cockroach, followed by a polygraph record showing the integrated electrical activity from the same nerve. B-E. Oscilloscope records from the same nerve. The roach had been decapitated twenty minutes before the record was made. Note the increased activity. F. Polygraph record of integrated nerve impulses from the phallic nerve of the same roach taken immediately after B-E. The film speed for the oscilloscope record was 25 cm./sec.; the polygraph tape moved 2.5 mm./sec.

thirty minutes (Fig. 2). Individual efferent fibers appear to follow their own burst frequency, giving a rhythmic and often complex pattern to the activity in the compound nerve. In most experiments after thirty minutes the burst pattern usually reaches its full development and remains constant for some hours. Occasional silent periods have been observed, however.

When an active extract of corpora cardiaca is applied to the nerve cord or injected into the head of a roach with an intact nerve cord, the effects of nerve cord transection or decapitation are mimicked (Fig. 3; compare with Fig. 3C, Roeder, Tozian and Weiant, 1960). The sequence of changes in the phallic nerve activity follows the same time course in the gradual development of bursts, and the magnitude of the response is very similar to that released by cord transection. The initial time lag when extract is applied is similar to that seen with decapitation. Desheathing portions of the abdominal ganglia and cord does not decrease this interval. The

only consistent difference is that activity produced by the corpus cardiacum extract is temporary, declining after about one hour. The bursting rhythm becomes irregular and the activity slowly dies down to its original level. If extract is reapplied to the cord every twenty minutes or so, the rhythmic efferent bursts may be maintained for several hours. These effects were observed in about sixty preparations.

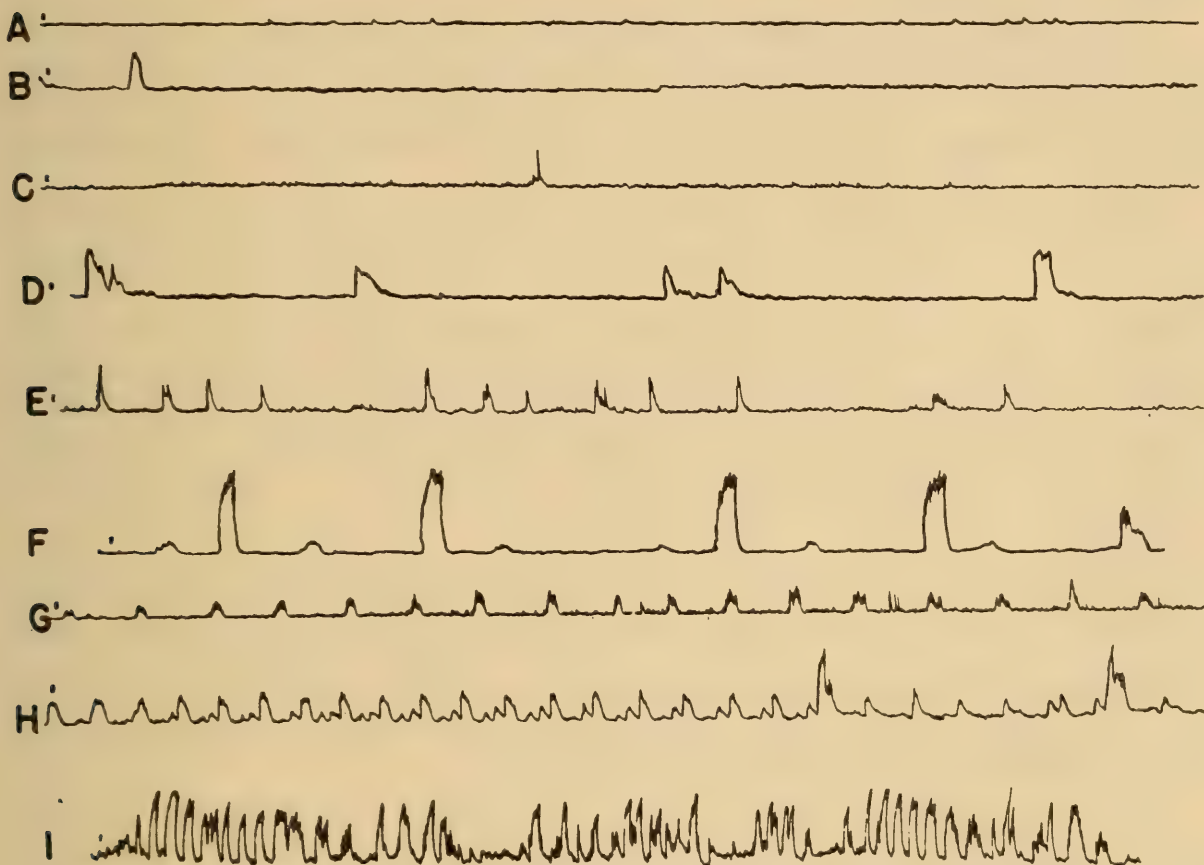


FIGURE 3. Polygraph records showing the integrated phallic nerve activity from the following roaches: A and B. Normal cockroaches. C. Roach with abdominal nerve cord exposed to corpus cardiacum extract, $\frac{1}{2}$ pair/0.01 ml., for 30 minutes (concentration below threshold). D. Roach with abdominal nerve cord exposed for 25 minutes to corpus cardiacum extract, 3 pairs/0.01 ml. from penultimate stage nymphs. E. Roach with abdominal nerve cord exposed to corpus cardiacum extract, 1 pair/0.01 ml., for 30 minutes. F. Roach with abdominal nerve cord exposed to corpus cardiacum extract, 2 pairs/0.01 ml. for seven minutes and (G) for 30 minutes. H. Roach which had had 3 pairs of corpora cardiaca in 0.02 ml. of saline injected into its head capsule 30 minutes before. I. Roach which had had 4 pairs of corpora cardiaca in 0.02 ml. of saline injected into its head capsule 35 minutes before.

The effects of extract concentration. The concentration of corpus cardiacum material necessary to produce the efferent nerve bursts varies both with the size and the species of the donor roaches and with the sensitivity of the test preparation. In general, the larger the adult size of the species, the more active were the extracts of its glands. In terms of their activity when tested on *Periplaneta*, extracts of *Blaberus giganteus* and *Blaberus craniifer* were about equally potent, while the glands of other species ranged down in activity in the following order: *Leucophaea maderae*, *Periplaneta americana*, *Byrsotria fumigata*, and *Diploptera punctata*.

The extract is always diluted by the blood of the roach so that quantitative information is difficult to obtain. An extract made from three or four pairs of corpora cardiaca diluted with 0.01 or 0.02 ml. of saline and injected into the head capsule produces pronounced increases in efferent fiber activity in the phallic nerves followed by rhythmic bursts of spikes. Extracts containing one or more pairs of corpora cardiaca per 0.01 ml. of saline when applied to the abdominal cord have similar effects (Fig. 3).

Control extracts were made from abdominal nerve cord tissue and applied in the same manner to the head ganglia and abdominal nerve cords of test roaches. Abdominal cord extract caused occasional transient increases in the activity of both the abdominal cord and the efferent nerves, but the regular and continuing efferent nerve spike bursts were never seen.

In experiments employing more concentrated extracts, containing up to three and a half pairs of corpora cardiaca per 0.01 ml., the initial time lag was unaffected, but the level of phallic motor nerve activity rose more rapidly than with less concentrated extracts and the final frequency attained by the efferent nerve spike bursts was higher. These rapid bursts, when integrated, appeared on the polygraph with a lower amplitude and a shorter duration than the slower-frequency bursts obtained with the less concentrated extracts (Fig. 3, F and G). The burst frequency about thirty-five minutes after the first application of extract provides a rough measure of the potency of the extract.

In four out of seven experiments in which concentrated extracts containing three and one-half pairs of glands per 0.01 ml. were applied to the abdominal cord, the phallic motor nerve activity was blocked after twenty to thirty minutes. This block was only partially reversible by washing. In fact, all attempts to wash out the effects of the corpus cardiacum extracts met with little success if the extract had been on the preparation for fifteen minutes or more. This may have been due in part to the difficulty of irrigating these preparations.

Activity in the abdominal nerve cord. The experiments of Özbas and Hodgson (1958) showed that nerve impulses in isolated roach abdominal nerve cords were blocked when the cords were immersed in extracts of corpora cardiaca. We have confirmed these observations and have also noticed a short period of excitation which seems to precede the block in such isolated cords. It has proved extremely difficult to block nerve impulses in abdominal cords with these extracts if the cords remain *in situ* with the cercal afferents intact and the head and thoracic portions of the cord still attached. When applied to a preparation in which the central nervous system is almost undamaged, the extracts usually cause a brief increase in the level of activity in the abdominal connectives. A tendency for some nerve cells to fire short trains of impulses becomes apparent after the extract has been on the cord twenty to thirty minutes. A period of depressed activity sometimes follows the period of excitation.

Simultaneous recordings made from abdominal connectives and from the phallic nerve do not reveal reciprocal changes in the levels of activity of the cord connectives and the motor nerves after decapitation or after application of extracts containing one to three pairs of corpora cardiaca per 0.01 ml. As the efferent nerve activity increases and bursts of nerve spikes begin to appear on the phallic nerves, the cord activity increases for a time and then usually returns to its previous

level. The cord appears depressed only when concentrations of extracts higher than three pairs per 0.01 ml. were used.

Nerve activity in the thoracic region. The changes induced by corpus cardiacum extracts in the activity of nerve 3 from the metathoracic ganglion and in the thoracic connectives were somewhat more complex than those obtained in the abdominal region. Of twenty-three experiments, fourteen showed increased activity followed by bursts similar to those observed in the phallic nerves. The change occurred twelve to twenty minutes after the extract was applied. In four cases the activity decreased and in four the efferent impulses were blocked. In these cases where the extract caused a temporary or partial block in cord and efferent nerves, twenty minutes were allowed for recovery, but the activity did not return to normal. However, if these roaches were then decapitated, there was an increase in the frequency of the active fibers in both the thoracic cord and nerve 3, and in one case there was also an increase in the number of fibers firing in nerve 3.

In one experiment there appeared to be no change in activity. A temporary decline or cessation of efferent activity was also noted at one period in four of the thirteen preparations which showed an over-all increase in activity and bursts. These changes induced by corpus cardiacum extract correspond closely with those following decapitation or transection of the thoracic connectives (Weiant, 1958).

Changes in activity recorded from electrodes placed on the nerve cord followed very closely the changes in nerve 3 described above. It seems probable that the increased efferent activity was picked up by the cord electrodes, and that this would have masked any decreased activity that might have occurred in the inter-nuncial fibers within the connectives.

In a few cases where the extract reached the exposed thoracic tissues, violent tremors developed in the flight muscles. Since all motor branches from the thoracic ganglia were severed, this suggests that the extract has a direct action on the thoracic muscles.

Abdominal pulsations often coincided with and obscured the thoracic nerve activity. If the abdomen was cut off at this time, there was some decline in the activity in nerve 3. However, this operation rarely brought the activity down to the level recorded previous to the application of extract. If severing the abdomen was followed by decapitation, a slight increase in the frequency of the activity in nerve 3 occurred.

Synaptic conduction. Six experiments were performed using the classic cercal nerve-giant fiber preparation (Roeder, Kennedy and Samson, 1947) to determine the effects of corpus cardiacum extracts on a sensory synapse. Extracts containing one to three pairs of glands per 0.01 ml. were applied or injected and left on the preparation for thirty to forty minutes. The only change which was observed was a slight lowering of the electrical threshold of the synapse in every case.

Comparison with DDT-toxin. Sternberg and Kearns (1952) have reported the presence of an excitor substance liberated from the nervous system of roaches poisoned with DDT. Four attempts have been made to collect this substance using a technique employed by Sternberg. The abdominal cord activity increased greatly after exposure to the saline containing the substance and many impulse trains were seen. No rhythmic spike bursts were observed on the phallic nerves,

however. It was concluded that the DDT-toxin was not the same as the principle in the corpus cardiacum extracts which causes phallic efferent nerve bursts.

CONCLUSIONS

The foregoing experiments demonstrate that, except for its reversible action, the extract of corpora cardiaca taken from several species of roaches mimics, in the American roach, removal of the inhibitory system as postulated by Roeder, Tozian and Weiant (1960). The simplest interpretation is that the corpus cardiacum extract suppresses the action of an inhibitory system, there being no evidence that it has any action on excitatory synapses.

If this is the case, a decrease in the activity of those fibers within the nerve cord that mediate the inhibition must occur in the presence of extracts of corpora cardiaca. This could not be detected by electrodes placed in contact with the outside of the nerve cord, although in the abdominal preparations the extract-induced increases in cord activity were transitory and insignificant compared with those occurring in the efferent fibers. Perhaps this is to be expected since the fibers comprising the inhibitory system may occupy only a small portion of the cord substance (Hess, 1958), and from the aspect of an external electrode they are probably shunted by many other fibers either unaffected by, or even released by, the blockage of the inhibitory system.

One perplexing observation in the earlier work (Roeder, Tozian and Weiant, 1960) was that the descending inhibitory system could not be blocked by KCl or by anelectrotonus, the only way of blocking it at that time being by cord transection. To this may be added the current observation that the inhibitory system may be inactivated not only by applying corpus cardiacum extract to its center or point of origin in the subesophageal ganglion, but also anywhere along its path from the head to the locally-originating neurones. It is difficult to visualize a classical nerve pathway operating in this manner, although no alternative mechanism can yet be proposed.

SUMMARY

1. Increased activity and regular bursts of efferent nerve spikes are seen on nerves IX and X from the cercal ganglion of roaches when extracts of corpora cardiaca are applied to the exposed abdominal cord or injected into the head capsule. Such motor nerve activity is usually inhibited in the intact animal by the presence of some influence arising in the subesophageal ganglion (Roeder, Tozian and Weiant, 1960). The corpus cardiacum extracts appear to suppress the action of this inhibitory center. The threshold concentration for this effect is approximately one pair of corpora cardiaca per 0.01 ml. of saline when the extract is applied to the abdominal cord, or three pairs injected into the head in about 0.01 to 0.02 ml. of saline. In decapitated male mantids a similar type of nerve fiber activity is believed to result in behavior associated with mating.

2. The corpus cardiacum extracts seem temporarily to increase the activity of the abdominal nerve cord in preparations with an intact central nervous system. When high concentrations of extract are used, the cord activity is occasionally depressed or blocked. The effects of the extract are only partially reversible by washing the preparation with saline.

3. The activity of certain efferent fibers in the thoracic region is similarly affected by corpus cardiacum extract, the action mimicking that produced by decapitation or cord transection.

4. Corpus cardiacum extracts have no significant effects on the transmission of impulses at the synapse between the cercal nerve and the giant fibers in an electrically stimulated preparation.

5. The DDT-toxin of Sternberg does not have the same effect on the roach nervous system as the active principle in extracts of corpora cardiaca.

6. The corpus cardiacum extracts may be preserved successfully by quick-freezing. Their activity is decreased by prolonged heating, but warming for five minutes at 90° to 100° C. leaves the potency unaffected. Such warming seems to increase by several hours the length of time that an extract will remain potent.

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ON THE HARDENING OF THE CHORION OF THE FISH EGG AFTER FERTILIZATION. III. THE MECHANISM OF CHORION HARDENING IN *ORYZIAS LATIPES*

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Shortly after fertilization or parthenogenetic activation, soft chorions of fishes are transformed into rigid structures mechanically non-elastic, and chemically resistant. This transformation has attracted much attention particularly in recent years (Nakano, 1956; T. S. Yamamoto, 1957; Ohtsuka, 1957; Zotin, 1958; Rothschild, 1958), and some interesting facts on substances participating in the phenomenon have come to our knowledge. However, the mechanism by which fish chorions are hardened is still a matter of speculation. In an earlier paper of this series (Ohtsuka, 1957), the possibility was suggested that in *Oryzias* egg the process underlying chorion hardening involves an oxidation process. This hypothesis seems to be provided further support in the present paper in which there are presented data on the effects of various chemical agents on the chorion hardening.

The author wishes to express his hearty thanks to Prof. I. Kawakami for his kind direction in the course of this investigation. He is also very grateful to Prof. N. Yoshii for his interest and encouragement.

MATERIAL AND METHODS

The ripe unfertilized eggs of the fresh-water fish, *Oryzias latipes*, were used as material. They were taken from matured females and kept in isotonic Ringer's solution. The overripe eggs of which chorions had slightly separated from the egg surface were discarded. The Ringer's solution used here had the following constitution: $M/7.5$ NaCl 100 parts + $M/7.5$ KCl 2.0 parts + $M/11$ CaCl_2 2.1 parts whose pH was adjusted to 7.3 by adding NaHCO_3 (T. Yamamoto, 1944). Sperm suspension was also prepared in Ringer's solution.

Treatments with chemical agents were carried out before or after fertilization at room temperature ($22-27.5^\circ \text{C}$). In the pretreatment, after some lengths of exposure, aliquots of the eggs were put back into Ringer's solution and then inseminated. Besides, in a few experiments insemination was done in the presence of reagents. The effect was examined with regard to chorion hardening. This is possible to judge indirectly from the degree of chorion elevation; it is generally accepted that the chorion is low when hardening is produced by the reagent whereas it elevates very highly under inhibitory condition. On this account the volume of elevated chorions was calculated 50 minutes after fertilization, according to T. Yamamoto's formula (1940). But the above criterion was found to have its

exceptions as described in the text. Therefore, in all the cases, chorions were torn with glass needles for the determination of their stiffness after volume measurement.

RESULTS

I. Oxidizing agents

The possibility that oxidation takes part in chorion hardening makes it of interest to test whether hardening is caused by means of oxidizing agents. Experiments were conducted on the action of potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate. All the agents were dissolved in Ringer's solution except for periodate. This was made up in Ca-free Ringer's solution to avoid precipitation by calcium.

When unfertilized eggs were subjected to these solutions (pH 7.3) for periods of 15 to 30 minutes, chorion hardening took place in the agents other than chromic acid. Such eggs with hard chorions exhibited no visible cortical changes during each treatment. Here it should be added that some of the eggs were partheno-

TABLE I

Effect of the presence of oxidizing agents on the chorion changes of cadmium-pretreated eggs at fertilization. Temp. 22.7° C.

Agents	Volume of chorion (cu. mm.)	Chorion hardening
No treatment	1.44 ± 0.018	—
Potassium ferricyanide (2×10^{-3} M)	0.99 ± 0.015	+
Sodium tetrathionate (5×10^{-3} M)	0.99 ± 0.020	+
Iodine in potassium iodide (2.5×10^{-4} M)	0.98 ± 0.017	+
Hydrogen peroxide (1.5%)	1.02 ± 0.014	+
Sodium periodate (4×10^{-4} M)	0.98 ± 0.012	+
Chromic acid (1×10^{-2} M)	1.40 ± 0.016	±
Potassium dichromate (2×10^{-4} M)	0.97 ± 0.011	+

genetically activated in both hydrogen peroxide and tetrathionate. In a chromic acid solution the chorion remained unchanged even after more prolonged exposure. It was found, however, that if the solution is acidic, chromic acid, though less effective, is capable of inducing hardening; at pH 6.6 it required a lapse of 60 minutes or more until the chorion became hardened. Then, Ringer's solution of chromic acid with this pH value was employed in the following experiments, but did not give any appreciable effect because of its feeble hardening ability. Treatments with oxidizing agents were carried out soon after fertilization. It was found that except in the case with chromic acid, all the other agents bring about the lowering of chorion elevation as a result of the acceleration of the hardening which follows fertilization.

Table I shows a typical result of experiments in which eggs immersed in a cadmium chloride-Ringer's solution (2.5×10^{-3} M) for 10 minutes were transferred to the solutions of oxidizing agents immediately following insemination in Ringer's solution. Cadmium treatment is known to abolish chorion hardening (Ohtsuka, 1957). It will be seen, however, that in these eggs except the chromic acid-treated ones, the chorions elevated were lower so that there developed a narrow perivitelline space.

It is of special interest that chromic acid is less effective than any of the other agents in inducing hardening. The discussion on this point will be presented later.

II. Reducing agents

Experiments were performed using the following reducing agents to determine whether their action on chorion hardening would be reverse to that of oxidizing agents: sodium sulfide, potassium cyanide, sodium thiosulfate, sodium sulfate, sodium thioglycolate, ammonium sulfate and potassium ferrocyanide. These agents were dissolved in appropriately diluted Ringer's solution so as to become osmotically equivalent to $M/7.5$ NaCl, and prepared in concentrations to contain the effective amount of calcium, since the deficiency of the latter blocks hardening of the chorion (Ohtsuka, 1957). The pH of all the solutions was made to 7.3 with HCl or NaOH.

Freshly inseminated eggs were put into each isotonic solution of the reducing agents. Here in both solutions of sulfide and cyanide the chorions were incapable of hardening, but such an effect of the remaining agents was observed only in a

TABLE II
Effect of the presence of reducing agents on the chorion changes at fertilization.
Temp. 24.1° C.

Agents	Pretreatment (min.)	Volume of chorion (cu. mm.)	Chorion hardening
No treatment	—	1.05 ± 0.014	+
Sodium sulfide ($2 \times 10^{-2} M$)	—	1.07 ± 0.017	—
Potassium cyanide ($3.3 \times 10^{-2} M$)	—	1.07 ± 0.012	—
Sodium thiosulfate ($6.6 \times 10^{-2} M$)	5	1.06 ± 0.023	—
Sodium sulfate ($6.6 \times 10^{-2} M$)	5	1.07 ± 0.018	—
Sodium thioglycolate ($9 \times 10^{-2} M$)	30	1.06 ± 0.015	—
Ammonium sulfate ($6.6 \times 10^{-2} M$)	5	1.06 ± 0.020	—
Potassium ferrocyanide ($4 \times 10^{-2} M$)	10	1.06 ± 0.013	—

few cases (*e.g.*, in 2 of 9 experiments with thiosulfate). If, however, fertilization was done in the presence of these agents with which the eggs had previously been treated for some periods (5–30 minutes), hardening always failed to occur. Reducing agents were found to be effective in high concentrations. One of the representative results is tabulated in Table II, where it can be seen that under the influence of reducing agents the chorions did not markedly elevate in spite of the inhibition of their hardening. And they were resistant to osmotic pressure of the perivitelline fluid. The factor inhibiting further elevation of the soft chorion in these circumstances is not known. On the other hand, it was impossible to prevent chorion hardening when eggs were inseminated in Ringer's solution after any length of pretreatments. Furthermore, the chorions once hardened could not be converted into a soft condition by treatments with reducing agents.

III. Sulfhydryl compounds

It has not yet been established on the nature of the chorion substrate undergoing hardening in fish eggs. The author (1957) was interested in SH groups,

TABLE III

Effect of 5 minutes' pretreatments with mercaptide-forming agents on the chorion changes at fertilization. Temp. 25.4° C.

Agents	Volume of chorion (cu. mm.)	Chorion hardening
No pretreatment	1.05 $\pm$ 0.014	+
Mercuric chloride (2.5×10^{-5} M)	1.47 $\pm$ 0.022	—
p-Chloromercuribenzoate*	1.48 $\pm$ 0.016	—

* Saturated in Ringer's solution.

anticipating that their reactivity might contribute to the chorion hardening. To elucidate this point, two series of experiments were undertaken, one with a mercaptide-forming agent and the other an alkylating agent. Preliminary study by a staining method on the composition of soft chorion seems to demonstrate the presence of SH groups (presumably protein bound-SH groups); no nitroprusside reaction was given, while it was stained with coloured SH reagent (method of Mescon and Flesch), even when this staining was applied after fixing the chorion with 2 per cent trichloroacetic acid.

In the first experiments, unfertilized eggs were placed for 5 minutes in two mercaptide-forming agents in Ringer's solution, mercuric chloride and p-chloro-

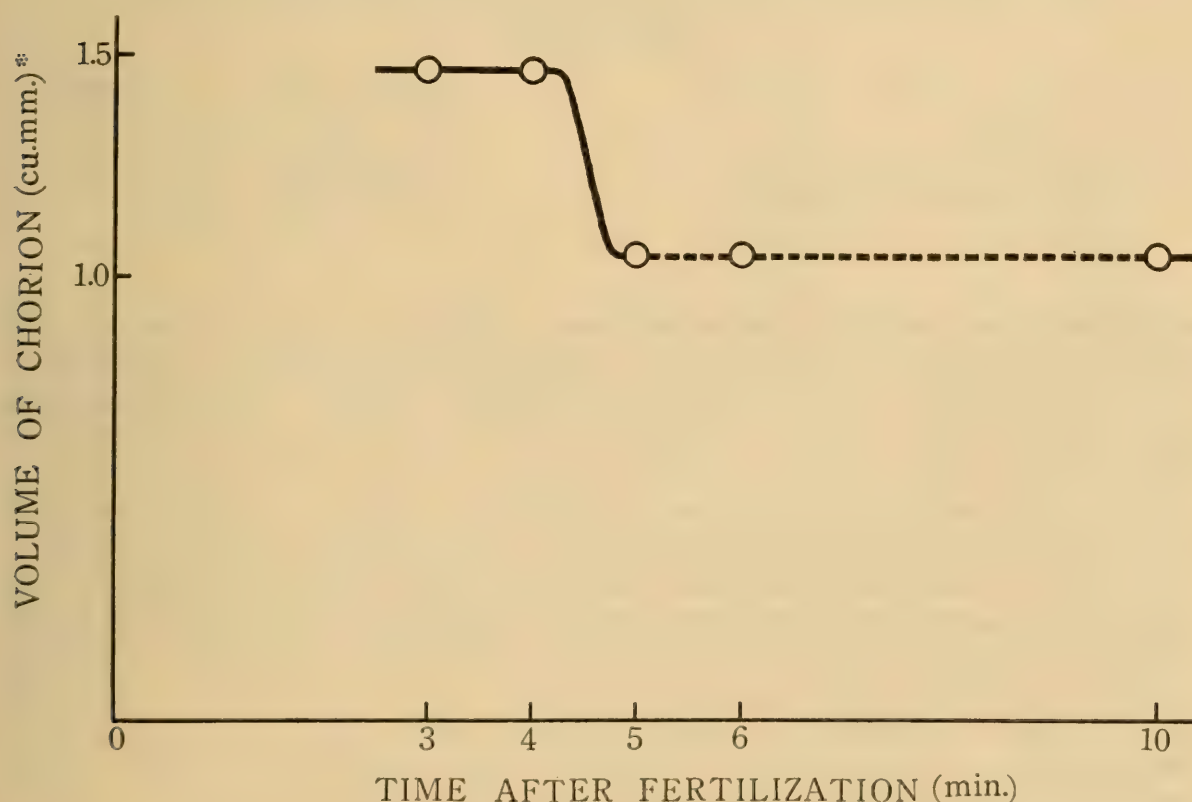


FIGURE 1. Changes in chorion volume in PCMB-Ringer's solution at different times after fertilization. Dotted line shows a period during which the chorion was burst by osmotic pressure of perivitelline fluid.

* Points (5, 10 and 15) of chorion volume in Figure 2 of the previous paper (Ohtsuka, 1957) should be corrected to 0.5, 1.0 and 1.5, respectively.

mercuribenzoate (PCMB). (Lapse of more than 5 minutes in these solutions caused the migration of cortical alveoli towards the animal pole without activation of the egg.) Insemination was carried out in Ringer's solution immediately after the pretreatment. In both cases, chorions were deprived of hardening and there occurred a marked elevation followed by bursting (Table III). This inhibition is due to the blocking of SH groups as shown in the following experiment. According to Anson (1945), the combination of mercury with SH groups can be removed by cyanide. Then, eggs which had been treated with mercaptide-forming agents for 5 minutes as above were consecutively transferred to potassium cyanide-Ringer's solution (3.3×10^{-2} M, pH 7.3) prior to insemination. When these eggs were fertilized in Ringer's solution after 5 minutes, reversal of the inhibition was observed. The chorion showed a normal degree of elevation, owing apparently to the occurrence of its hardening.

The failure to harden was also produced by exposing eggs to each mercaptide-forming agent after insemination, and their inhibitory effect could similarly be cancelled upon subsequent treatment with potassium cyanide. Figure 1 represents the results of a typical experiment with PCMB at 25.8° C. If PCMB was applied within 10 minutes after fertilization, further hardening did not proceed

TABLE IV
Effect of the presence of alkylating agents on the chorion changes at fertilization.
Temp. 26.2° C.

Agents	Volume of chorion (cu. mm.)	Chorion hardening
No treatment	1.05 ± 0.012	+
Iodoacetic acid (3.3×10^{-2} M)	1.48 ± 0.017	—
Chloroacetophenone* (0.05%)	1.47 ± 0.024	—

* Saturated in Ringer's solution.

and the elevated chorions retained softness. It should be noted, however, that when this treatment was made about 5 minutes after fertilization or later, such soft chorions became incapable of elevating highly. Unlike the cases of reducing agents described above, they were burst by an osmotic pressure of the perivitelline fluid.

In the next experiments, eggs were subjected to treatment with iodoacetic acid or chloroacetophenone. These alkylating agents were made up in Ringer's solution (pH 7.3). It was found that either short treatment of unfertilized eggs is not inhibitory to chorion hardening when they are subsequently inseminated in Ringer's solution. If the duration of these exposures was long, in the case of chloroacetophenone fertilized eggs underwent cytolysis and the iodoacetic acid-treated eggs lost their fertilizability. When, however, both agents were applied after insemination, the chorions failed to harden. Eggs exposed to alkylating agents soon after fertilization formed considerably high chorions (Table IV).

IV. Urea and lithium bromide

The above experiments clearly indicate that SH groups primarily participate in hardening of the chorion. They might be expected to be available as a possible

donor of hydrogen bonds. If this is really the case, the latter thus given would be responsible for hardening. According to Nakano (1956), the hydrogen bonds are concerned in the mechanical properties of the chorion of *Oryzias*. Attempts were thus made with urea and lithium bromide, which are known to cleave hydrogen bonds. The maximum concentrations at which they were tested were prepared in $3.3 \times 10^{-2} M$ both in Ringer's solution. Eggs were treated before or after fertilization. But neither reagent prevented chorion hardening, even in those cases where unfertilized eggs were exposed for 60 minutes and then inseminated therein. Thus it seems that hydrogen bonds play no role in giving rise to a chorion hardening.

V. Aldehydes

In a staining study, the soft chorion was further found to be periodate-Schiff-positive (Lillie's method), which was blocked by the acetylation technique of McManus. From this result it is evident that a polysaccharide having α -glycol groups impregnates the chorion and produces aldehydes on oxidation (*cf.* Lison, 1953). The question arises whether such aldehydes take part in hardening of

TABLE V

Effect of the presence of aldehydes on the chorion changes of cadmium-pretreated eggs at fertilization. Temp. 27.3° C.

Agents	Volume of chorion (cu. mm.)	Chorion hardening
No treatment	1.48 ± 0.017	—
Formaldehyde (0.03%)	0.97 ± 0.014	+
Acetaldehyde (0.5%)	0.98 ± 0.018	+
Acrolein (0.05%)	1.01 ± 0.012	+
Benzaldehyde (0.2%)	0.99 ± 0.015	+

the chorion. To clarify this point, the effect of aldehydes, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde, was investigated. Ringer's solutions of these agents were adjusted to pH 7.3 by adding NaOH.

The results obtained were essentially the same as those with oxidizing agents noted already. The chorions became hardened without egg activation in the presence of the agents other than acrolein. In the latter solution, almost all eggs were parthenogenetically activated 2–3 minutes after their immersion and there took place faster chorion hardening than in the control. Table V illustrates that aldehydes bring about the formation of low chorion when cadmium-pretreated eggs are treated after fertilization in the same way as in the cases of oxidizing agents.

DISCUSSION

As shown in the foregoing pages, the chorion fails to harden under the influence of certain reducing agents. Very high concentrations of the agents are characteristic of this inhibition, suggesting the existence of an antagonistic force responsible for hardening. It has been reported in *Oryzias* egg that a phospholipid in the perivitelline space, which is originated from the cortical layer itself following fertilization or artificial activation, is of prime importance in giving rise to the

chorion hardening, the action of which is regarded as oxidative in nature (Ohtsuka, 1957). Therefore it is most probable that reducing agents inhibit hardening by interfering with this oxidative effect. On the other hand, the oxidizing agents, potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate, were found to have the hardening capacity. The effectiveness of these agents in hardening the chorion is probably due to oxidation. We shall return to this aspect later.

Similar results have been obtained with sea urchin eggs by Motomura and Hiwatashi (1954), who tested the effects of various chemicals on the hardening of the fertilization membrane, and found that the action of reducing agents is opposite to that of oxidizing agents; the former inhibits membrane hardening whereas the latter accelerates it. They offered an explanation that hardening is concerned with polymerization to which both agents are sensitive in a reverse manner. But, as just discussed, the present results can be reasonably interpreted from an oxidative point of view. Still this does not preclude the possibility of polymerization in the hardening of the chorion.

In view of these considerations, it may be allowable to conclude that the oxidation process is involved in the process whereby *Oryzias* chorion is converted into rigid structure. And it appears that it takes part in the initial stage of the hardening process, as is indicated by the fact, for example, that the chorion became hardened even when treatment with sodium periodate was carried out for 10 minutes, during which no changes were visible, and then kept in Ringer's solution. This conclusion is, however, contradicted by Nakano's observation (1956) that colloidal substances, such as gum arabic, gum tragacanth, albumin and gelatin, are capable of hardening *Oryzias* chorion. These substances are unfortunately non-oxidative in chemical nature, their mechanisms remaining obscure.

In a more recent study, Zotin (1958) has found a substance indispensable for the chorion hardening in the perivitelline space of some salmonid eggs. According to him, this substance, which was termed the hardening enzyme, is secreted from the cortical layer, but not from the alveoli present there, upon fertilization or activation, and plays an active role in hardening the chorion. Thus there is a considerable resemblance between phospholipid in *Oryzias* egg and the hardening enzyme of salmonid eggs, particularly with respect to their source and behaviour. Furthermore, Zotin described that hardening of the chorion in salmonids, which is due to polymerization of some substances, is blocked by oxygen deficiency.

It has been shown in the present paper that the SH groups (presumably protein bound-SH groups) of the chorion are essentially necessary for hardening, while their role bears no relation to their possible capacity to give hydrogen bonds. In this connection it should be emphasized that the first three of the effective oxidants noted above, potassium ferricyanide, sodium tetrathionate, and iodine, all oxidize SH groups (Anson, 1945). A similar action, though less reliable, is known about hydrogen peroxide (see Hirai, 1957) capable of hardening as well. Hence these strongly suggest an oxidative conversion of SH into S-S; the latter thus formed may be responsible for chorion hardening. This change must proceed beyond the reversible stage, because a hard chorion is not returned to the soft condition by reducing agents so far as the present study is concerned. Additionally, there is evidence that a chorion polysaccharide having α -glycol groups participates

in hardening. Sodium periodate, another effective oxidant, does change all the available α -glycol groups to aldehydes (*cf.* Lison, 1953). Then, if such a type of oxidation is involved in the chorion hardening, it is worth pointing out that chromic acid likewise produces aldehydes in oxidizing the polysaccharide but it seems without action on α -glycol groups (*cf.* Lison, 1953); this may be the reason why chromic acid is less effective in inducing hardening in spite of its strong oxidative capacity. It is therefore likely that in hardening α -glycol groups are subject to oxidation, resulting in the formation of aldehyde groups. Favoring this idea is the fact that the chorions harden by treatments with aldehyde, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde. Thus it may be said that two substances of the chorion, a protein containing SH groups and a polysaccharide which has α -glycol groups, form a complex undergoing hardening.

From the above account it follows that the reactivities of both sulfhydryl and α -glycol groups contribute to the hardening of the chorion. There must occur some profound changes in these two groups, especially with regard to their, and perhaps reciprocal, oxidative reactions. However, an alternative possibility remains, based on the following facts: According to the present data, SH groups become responsible for chorion hardening about 10 minutes after fertilization. It was further shown that when PCMB, which attacks SH groups, is applied about 5 minutes after fertilization, the chorion retaining softness is incapable of elevating very highly, being burst by osmotic pressure of the perivitelline fluid. These observations reveal some preceding change, probably of polysaccharide, and favor the assumption that oxidation concerns first the α -glycol groups only and then it is followed by that of SH. Significant in this respect is a hardening effect of certain aldehydes mentioned already, and moreover evidence is available that aldehydes are able to combine with SH groups (Schubert, 1936), which latter still remain capable of being oxidized (Anson, 1945).

Thus it may be suggested that hardening is due to the combination of at least two factors in the chorion, an SH group, and an aldehyde produced by oxidation of α -glycol group. And the former would undergo oxidation to S-S. There might be an oxidation polymerization through these disulfide bridges. Very interesting is that the reaction between SH groups and aldehydes is related to a skin tanning (Hirai, 1957). Furthermore, it should be mentioned that potassium dichromate capable of hardening is an excellent tanning agent. Considering these, a tanning reaction appears to take place in the chorion hardening. Rothschild (1958) proposed that fish chorions are hardened by the tanning effect of substance which is discharged from the cortical alveoli at fertilization or parthenogenetic activation. The positive effect of the alveolar substance on chorion hardening has been suggested by Nakano (1956) in *Oryzias* and by T. S. Yamamoto (1957) in *Oncorhynchus*. But the unpublished data on *Oryzias* egg show that hardening is independent of the alveolar substance.

SUMMARY

1. Oxidizing agents, such as potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate, cause hardening of the chorion. Chromic acid is less effective than any of the other agents.

2. In the presence of reducing agents, such as sodium sulfide, potassium cyanide, sodium thiosulfate, sodium sulfate, sodium thioglycolate, ammonium sulfate, and potassium ferrocyanide, the chorions fail to harden.

3. Treatment of unfertilized eggs with mercuric chloride or p-chloromercuribenzoate induces the inhibition of chorion hardening when they are subsequently inseminated in Ringer's solution. The same result is also obtained in the cases where both agents are applied after fertilization. Either inhibitory effect is removed if the treatment is followed by that with potassium cyanide.

4. Iodoacetic acid and chloroacetophenone likewise inhibit hardening when eggs are treated after insemination.

5. Neither urea nor lithium bromide has any effect on the chorion hardening.

6. Aldehydes, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde, are capable of hardening the chorion.

7. Soft chorion is impregnated with a protein containing SH groups, together with polysaccharide which has α -glycol groups.

8. It is concluded that the oxidation process is involved in the mechanism of chorion hardening. It is suggested that hardening is due to combination of SH groups with the aldehydes produced by oxidation of α -glycol groups.

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LOW TEMPERATURE BLOCKAGE OF MOLTING IN *UCA PUGNAX*

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The fiddler crabs, members of the genus *Uca*, have been the favorite animals for the experimental investigation of molting in brachyurans by American comparative endocrinologists. The facility in collecting and holding these sand and mud flat burrowers and the simplicity of inducing proecdysis by bilateral eyestalk removal have resulted in use of these crabs and this technique by many investigators during the past two decades. Casual note has been made of the variation in proecdysis duration observed, some of which has been attributed to differences in ambient temperatures. Presumably because of limited facilities, no systematic study has been made of the effect of temperature on either proecdysis duration or proecdysis initiation.

In a preliminary observation incidental to a previously reported study of the endocrinological basis of molting (Passano, 1953) a group of 20 male *Uca pugnax* had both eyestalks removed in early winter and were then kept for 35 days in individual bowls at room temperatures diurnally fluctuating between 14° and 21° C. There was a high mortality rate, but of the 8 crabs surviving, none molted. When put in a 27° constant temperature room, three crabs molted after 3, 7, and 8 days. Since eyestalkless *U. pugnax* kept at 23.5° C. showed an average proecdysis period of 19.3 days (Passano, 1953), it appeared as though a 5° reduction in temperature might block or markedly delay a forced ecdysis initiated by eyestalk removal (*i.e.*, by extirpation of the X-organ sinus gland complex).

The experiment reported here has two parts: 1) exposure to a constant test temperature for 23 days, following elimination of the molt-inhibiting hormone (MIH) by bilateral eyestalk removal, and 2) a post-treatment period at the optimum temperature for induced proecdysis, to measure the extent of the previous temperature-induced proecdysis block.

MATERIALS AND METHODS

Approximately 600 male marsh fiddler crabs, *Uca pugnax* (S. I. Smith), were collected from a salt marsh near New Haven, Connecticut, in late April (water temperature 12° C., air temperature 8°–15° C.) and acclimatized to 15° for one week. Four hundred animals of uniform size were then selected, discarding any whose major chela was missing or undersized, or which had autotomized more than one walking leg, so that the selected crabs had approximately the same blood volume. One eyestalk was removed by cutting across the arthrodial membrane at the base of the eyestalk with fine scissors and each animal was forced to autotomize one of its walking legs, usually the fourth leg on the side opposite to the major chela. The following day the other eyestalk was removed in a like manner. Twelve hours later the surviving eyestalkless animals were divided into ten groups

of 38 crabs each, hereafter called Groups A, B, C . . . J, with the use of a table of random numbers. Subsequent measurement showed that these groups were homogeneous in size distribution with a standard length mean, and standard deviation of the mean, of 18.7 ± 1.2 mm. (Table I). Each animal was put in an individual covered glass dish (or "fingerbowl") with sufficient water (1:1 Long Island Sound Water:tap water; salinity 14‰) to cover the maxillipeds. The water was changed on the sixth, twentieth and thirty-third days after eyestalk removal. Each group was placed in a separate temperature-controlled room (Table I) and kept there for the temperature treatment period of 23 days. Those rooms closest to ambient temperatures, 20° and 22°, showed the largest fluctuations in air temperature, but these rarely exceeded 1° and were rapid and cyclic. A control group of 19 crabs was placed at the warmest temperature after forced

TABLE I

The effect of exposure to different temperatures on ecdysis by eyestalkless Uca

Group	Number (after initial mortality)	Size (standard length, mm.)	Temperature treatment (23 days)			Post-treatment (20 days at 29°)		Total	
			Temp. ° C.	% molt	% re- maining	% molt	% re- maining	% molt	Mean proecdysis duration and S.D.
A	37	18.7	13.1	0	92	46	14	46	$37.4 \pm .85$
B	37	18.6	15.3	0	86	43	26	43	$36.8 \pm .79$
C	37	18.5	16.3	0	97	54	19	54	35.7 ± 1.00
D	37	18.6	18.2	0	97	59	16	59	$33.4 \pm .97$
E	38	18.7	20.0	0	100	58	13	58	31.8 ± 1.05
F	36	18.5	22.2	25	72	31	8	56	24.8 ± 1.15
G	37	18.8	24.4	73	16	11	0	84	$19.3 \pm .61$
H	35	18.5	26.7	76	17	6	0	81	$17.4 \pm .63$
I	37	18.5	29.4	89	5	0	0	89	$14.8 \pm .73$
J	37	18.8	32.2	87	0	0	0	87	$14.4 \pm .49$
Controls	19	19.0	32.2	0	53	0	11	0	—
	387	18.7 ± 1.2							

autotomy of one walking leg, but as expected, none molted nor showed signs of entering proecdysis. Since the experimental animals were blinded, no regular photoperiods were given. The crabs were not fed, inasmuch as it was impossible to insure equal uptake in the different temperature groups or by individual crabs at the same temperature.

Mortality during the temperature treatment period of the experiment was light. Deaths during the first week (3.2%) are considered to be due to operational mortality and are not included in the tabulated results.

Although some animals successfully completed ecdysis following the experimental treatment, the largest number died during ecdysis or were unable to withdraw all of their appendages. Any crabs dying in Stage E (Drach, 1939) were scored as molted, while those few dying in Stage D₁ were scored as having molted the following day. No animals were kept after ecdysis.

After 23 days, those crabs remaining were examined for evidence of proecdysis initiation. The regeneration of the previously autotomized fourth walking leg was used as an indicator (Bliss, 1956; Jyssum and Passano, 1957). The animals were then slowly equilibrated to 29.4° and kept there for a further 20 days to determine what effect, if any, the initial temperature treatment had had on proecdysis initiation. When the experiment was ended, 43 days after removal of the second eyestalk, 8.5% of the animals remained.

RESULTS

Following elimination of the MIH by bilateral eyestalk removal, a total of 242 of the 368 animals (66%) eventually reached or completed ecdysis. None of the control crabs molted. Table I and Figures 1 and 2 show the differential effect of

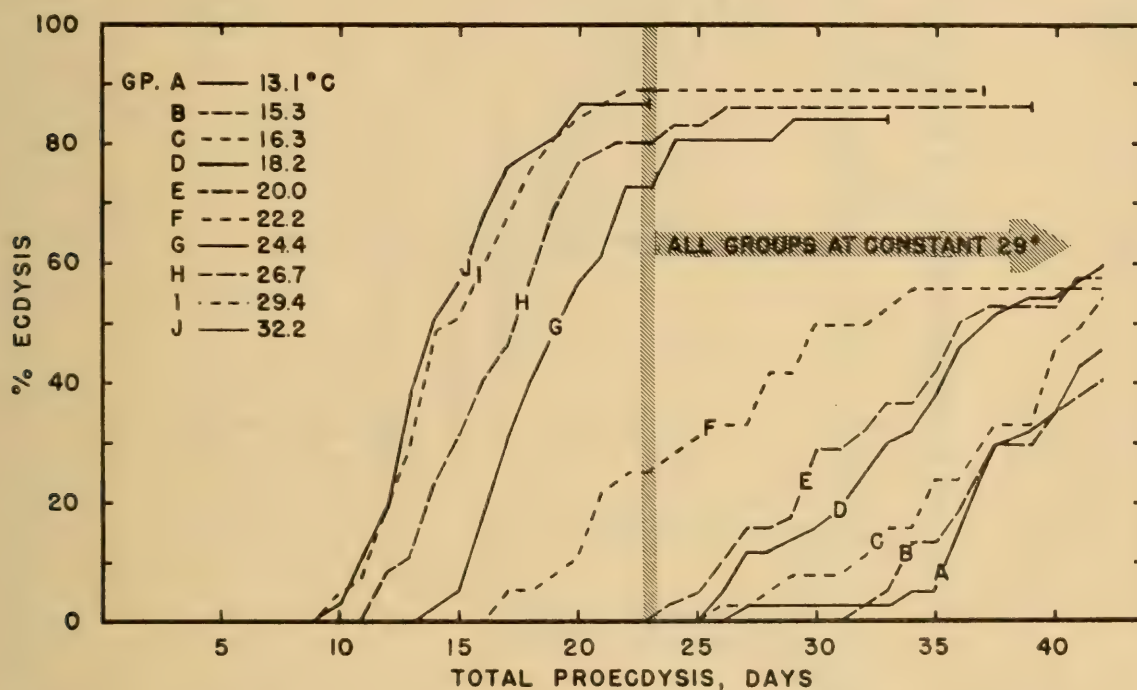


FIGURE 1. Cumulative percentages of *Uca* molting in each experimental group following eyestalk extirpation.

temperature on average proecdysis duration, the per cent completing proecdysis and the uniformity of response within each group. The low values of the standard errors of the mean for the proecdysis duration of each group are noteworthy.

1. Low temperature treatment, Groups A, B, and C

Exposure to constant low temperature in the range from 13.1° to 15.3° C. blocks proecdysis initiation almost completely, in spite of elimination of the MIH. With a single exception in the group kept at the lowest temperature, none of the Group A or Group B animals initiated discernible proecdysis during the temperature treatment period. Only 14% of these crabs showed basal limb bud (Bliss, 1956) regeneration of their autotomized pereopod, itself a proecdysis-independent process (Jyssum and Passano, 1957). Again with the single exception already

noted, none reached the ecdysis stage, Stage E (Drach, 1939), in less than 9 days after being placed at 29.4° (Fig. 1). The mean proecdysis duration in the post-treatment warm temperature for Groups A and B combined was 14.1 days with a standard error of the mean of ± 0.48 days. This is not significantly different from the value for Group I crabs which were put directly at 29.4° after eyestalk removal (Fig. 2, shaded bar), although it is shorter.

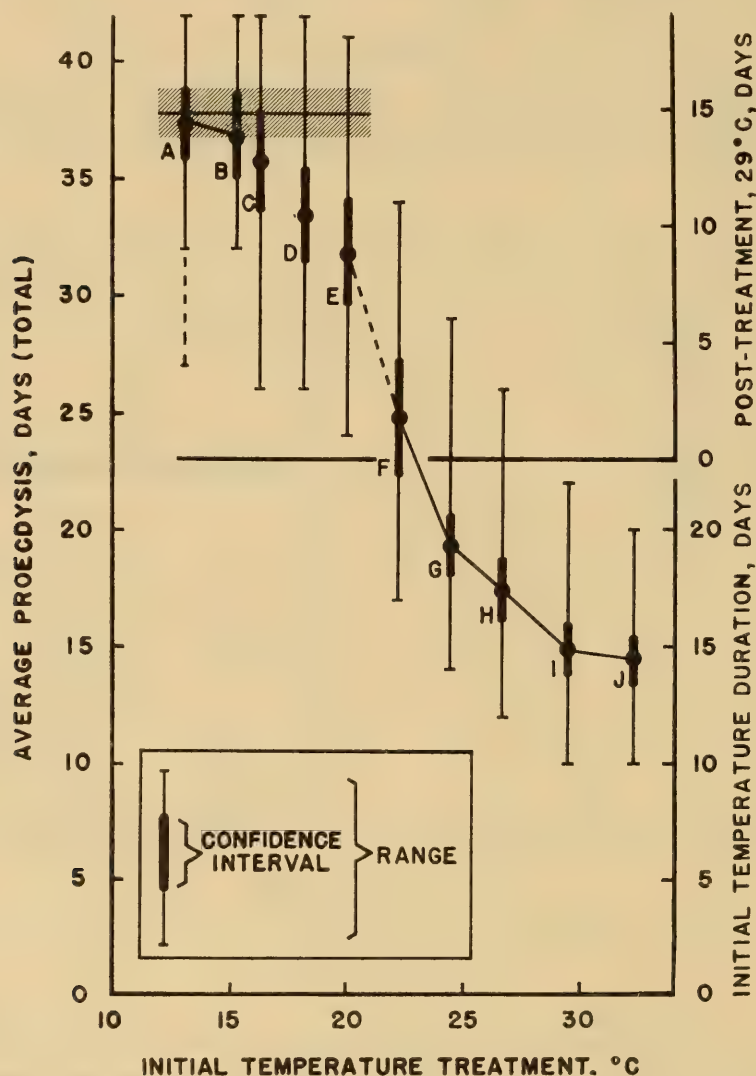


FIGURE 2. The effect of various initial temperature treatments on the proecdysis duration of eyestalkless *Uca*. The shaded horizontal bar represents the confidence interval (13.7–15.9 days) for animals put directly into 29.4° C., transposed to the upper, post-treatment, portion of the graph.

Group A's exceptional animal molted on the fourth day of post-treatment warm temperature. It showed no limb regeneration whatsoever. Since it is probable that this individual had already commenced proecdysis prior to the beginning of the experiment, the increase in the extremes of proecdysis duration caused by this aberrant animal is shown by a dashed line in Figure 2.

Group C, kept at 16.3° for the temperature treatment period, also shows a

post-treatment proecdysis duration, 12.7 ± 1.0 days, insignificantly different from that of Groups A and B. However, half the crabs showed some basal limb bud regeneration after 23 days at 16.3° and one crab, which molted six days later after being raised to 29.4° , had a "premolt limb bud" (Jyssum and Passano, 1957) at that time. The other three animals which molted prior to the tenth post-treatment day again showed no limb regeneration at all.

2. High temperature treatment, Groups G, H, I and J

There was no discernible proecdysis blockage in the groups of animals kept at 24.4° or above (Figs. 1, 2). The two groups kept at the highest temperatures, J (32.2°) and I (29.4°), had average proecdysis durations of 14.4 ± 0.49 days and 14.8 ± 0.73 days, respectively, the slight difference being without statistical validity. The somewhat greater standard deviation of Group I is due to a slight skew of the distribution curve towards the longer proecdysis (Fig. 1). In neither case did any molting occur prior to 10 days, nor 22 days after, removal of the second eyestalk. Molting occurred in 88% of Groups I and J, and of these 94% regenerated the autotomized pereopod.

Groups H (26.7°) and G (24.4°) also showed a nearly uniform molting response but here proecdysis duration was significantly increased ($P < 0.01\%$; $P < 0.04\%$). As in the groups kept at the highest temperatures, nearly all regenerated their autotomized walking leg.

3. Intermediate temperature, Groups D, E, and F

None of the crabs kept at 18.2° (Group D) or 20.0° (Group E) reached ecdysis during the temperature treatment period, but examination of the regenerating limb buds showed that at least 50% were in proecdysis. During the post-treatment period at 29.4° , approximately half of those crabs which eventually did reach ecdysis had done so before the temperature-blocked animals of Groups A, B and C began to molt (Fig. 1). Nearly all of the Group F molts (22.2°) had molted by this time.

The largest standard deviation from mean proecdysis duration occurred within these intermediate temperature groups, in Group F. Many of those animals in which proecdysis appeared to be blocked failed to survive the 20-day post-treatment period at 29.5° (Table I).

DISCUSSION AND CONCLUSIONS

Molting after bilateral eyestalk extirpation is due to elimination of molt-inhibiting hormone (MIH) of the medulla terminalis ganglionic X-organ (Bliss, 1953; Passano, 1953) and consequent formation and release of molting hormone (MH) by the Y-organ (Echalier, 1959). MH then causes the sequence of morphogenic and biochemical events collectively termed proecdysis, ecdysis and postecdysis.

The results reported here show that proecdysis duration is strongly temperature-dependent. In *Uca pugnax* a temperature between 29° and 32° gives the shortest average proecdysis duration. Temperatures above this may be deleterious to this

crab, since most of the normal control animals failed to survive the 42 days of this experiment, but it is equally plausible that these animals starved to death. Temperatures somewhat below 29° still permit molting but the proecdysis period is progressively lengthened, doubling in duration when the temperature falls from 30° to 20° .

Temperatures of 15° or below effectively block proecdysis. A comparison between either Group A or Group B and Group I shows that mean proecdysis duration at 29° is nearly the same whether or not the eyestalkless animals had first been kept at 15° for three weeks (Fig. 2). No appreciable proecdysis occurs during this period in these groups. The low temperature treatment did reduce the total percentage molting to one-half its original value (Table I). This is presumably due to the continuous depletion of organic reserves during the three weeks of low temperature treatment starvation. If the experimental animals had been fed, this decrease in the percentage of the group reaching ecdysis would probably have not occurred.

At some temperature between 15° and 22° proecdysis may either fail to commence, or else may be initiated but proceed slowly, after eyestalk removal. The lengthened mean proecdysis durations of the intermediate temperature groups, D (18°) and E (20°), are due in part to proecdysis blockage in some of the crabs. After three weeks at the treatment temperatures, 42% of the 18° animals and 39% of the 20° animals showed no signs of proecdysis initiation. The remaining crabs had initiated proecdysis but its duration was lengthened by the "low" temperature. It is thus to be expected that these intermediate temperature groups are the most heterogeneous in their response of all the temperature groups tested (Fig. 2).

It is scarcely surprising to find an inverse correlation between temperature and proecdysis duration. It is unusual to find that a temperature of 15° or even higher can block completely an animal's growth, even though this species experiences lower temperatures through much of its life cycle. Since it can survive at these lower temperatures for long periods of time, it seems probable that some key reaction, required in proecdysis initiation, is being blocked. The first half of the proecdysis requires MH, since crabs deprived of their Y-organs are permanently blocked in their intermolt stage (Echalier, 1959). Low temperatures might thus prevent the Y-organs from responding to the elimination of the MIH following eyestalk removal. Alternatively, these glands might form and release into the circulation adequate amounts of MH, but the target tissues might be unable to respond to the MH rise. Yet neither of these explanations accounts for the nearly complete correlation of proecdysis blockage and basal limb bud regeneration blockage found in these experiments. Although the main part of limb regeneration occurs only during proecdysis, so that regenerate size is a good index of proecdysis stage (Bliss, 1956), the initial or basal outgrowth of the regenerating limb is independent of Y-organ MH (Jyssum and Passano, 1957) and, unlike proecdysis initiation, is not photosensitive (Bliss, 1956). Therefore if the temperature-dependent proecdysis blockage found here is blocking Y-organ MH formation or activity, some other temperature-dependent step must be blocking basal limb bud formation in these starved animals.

Although a few of the animals which molted in this experiment failed to show

any limb regeneration, 92% did regenerate the autotomized walking leg. Perhaps temperatures of 15° or below limit some metabolic process common to both proecdysis and basal limb bud formation. One of the initial events in proecdysis is the mobilization of hepatopancreas metabolic reserves (Bliss, 1953; Passano, 1960), noted after eyestalk removal by a rise in oxygen consumption (Edwards, 1950; Bliss, 1953) and a fall in R.Q. (Bliss, 1953). Stored fats are utilized for proecdysis integument growth and mineral mobilization (Renaud, 1949). The basic metabolic requirements of the animal, as well as basal bud regeneration, must require storage depletion as long as animals remain unfed. Perhaps the low temperatures prevent sufficient mobilization of hepatopancreas reserves for basal limb regeneration and proecdysis initiation, as well as basal metabolism. It is possible that such mobilization is normally controlled by an eyestalk hormone in the intact animal at higher temperatures, since eyestalk removal causes a rise in oxygen consumption whether or not the Y-organs are intact (Passano, unpublished). It is not known whether the same rise in O₂ consumption follows bilateral X-organ extirpation in the Y-organless animal; if so, the MIH may be controlling proecdysis initiation by limiting the fat depot mobilization necessary for MH synthesis.

An alternative explanation might be developed from the hypothesis that all crustacean growth processes are controlled in an identical manner (Bliss, 1956). Thus basal limb growth would be under MH control, but this MH would originate in some extra-Y-organ site such as the individual limb bud blastema. It would support this alternative if it were found that Y-organless crabs could be forced into proecdysis by initiating mass basal limb bud regeneration. Multiple regeneration in normal crabs can lead to ecdysis even though environmental conditions are unfavorable (Bliss, 1956), but such treatment might activate their intact Y-organs. If such extra-Y-organ sources of MH occur, then low temperatures could be blocking MH formation or activity, irrespective of the hormone's origin.

Uca (including *U. minax* (Le Conte) and *U. pugilator* (Bosc) in addition to the species used here) occurs on the East Coast of the United States as far north as Cape Cod, but does not commonly occur north of the Cape (Rathbun, 1918). The notable discontinuity at Cape Cod in the littoral fauna is primarily due to the cooler summer water temperatures of the Gulf of Maine, yet the temperature experienced by adult *Uca* must be primarily determined by ambient air temperature rather than water temperatures. Since there is no marked air temperature differential between the littoral areas north and south of Cape Cod, it seems likely that the temperature limit on *Uca* acts on growth of its pelagic larvae rather than on post-larval growth. Perhaps, then, the significance of the proecdysis temperature block found in these experiments is that it reflects an identical limitation of proecdysis initiation occurring in *Uca* larvae, so that larvae hatching in the cooler Gulf of Maine waters are unable to grow because they are unable to molt. Caution must be maintained, however, in attributing species limits to simple temperature effects (Moore, 1958).

SUMMARY

1. A uniform population of male *Uca pugnax* was used in studying the effect of 10 temperatures on proecdysis resulting from eyestalk removal.
2. Proecdysis duration is shortest at 29° to 32° C. and lengthens significantly at lower temperatures.

3. Proecdysis initiation is markedly temperature-sensitive. At 15° or below initiation is completely blocked; at 15° to 20° a substantial proportion of the crabs fail to begin proecdysis.

4. Temperatures which block proecdysis initiation also block basal limb bud regeneration (a molt-independent growth process). It is hypothesized that a metabolic event common to both processes is being blocked.

5. The physiological and ecological significance of the proecdysis temperature block is considered.

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THE DEPOSITION OF SKELETAL STRUCTURES IN THE CRUSTACEA. I. THE HISTOLOGY OF THE GASTROLITH SKELETAL TISSUE COMPLEX AND THE GASTROLITH IN THE CRAYFISH, *ORCONECTES* (CAMBARUS) *VIRILIS* HAGEN—DECAPODA¹

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Little is known regarding the basic mechanisms by which certain cells and tissues participate in synthesis and calcification of organic matrices. The gastrolith discs and the branchial exoskeleton provide particularly useful experimental material in the Crustacea for such studies. Attention, in this paper, will be confined to the histological study of the gastrolith discs of the fresh-water crayfish, *Orconectes virilis* Hagen.

The paired gastrolith discs are composed of the cuticular lining of the stomach, the thickened gastric epidermis, and the underlying sub-epidermal connective tissue. They are located in the anterior lateral walls of the cardiac stomach of the crayfish. Their usefulness as sites of activity for the study of cellular processes involved in the synthesis and calcification of organic matrices is evident when it is realized that the epidermis of these modified portions of the stomach wall becomes competent to synthesize and calcify the gastrolith matrix preceding molt. This activity culminates at the end of the premolt period in the formation of hard calcified disc-shaped gastroliths which lie in a sac or pouch now formed between the epidermis and cuticular lining of the stomach. At the same time gastrolith formation occurs, the epidermis of the exoskeleton is participating in resorption of mineral and organic constituents. At the molt, the fully formed gastroliths are shed with the old stomach lining into the stomach. Following molt, these gastroliths are gradually broken down and resorbed. Some of their mineral constituents are resorbed by the gastrolith epidermis and hepatopancreatic epithelium (Travis, in preparation). These mineral constituents are conveyed by the blood to the epidermis underlying the exoskeleton of other areas and are re-used in synthesis and calcification of their organic matrices.

While the description of the presence of gastroliths before molt and their gradual disappearance following molt has been given by a number of authors (Réaumur, 1712; Chantran, 1874a, 1874b; Braun, 1875; Huxley, 1879; Herrick, 1895; Irvine and Woodhead, 1889; Husson, 1952; and others), only certain aspects of the histology of the gastrolith discs have been described by Braun (1875) for the European crayfish, *Astacus fluviatilis*, and by Herrick (1895) for the American lobster, *Homarus americanus*. The histological changes have neither been described with reference to stages of the molting cycle nor to the synthesis of the non-calcified

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skeletal components of the gastrolith discs, and only with brief reference to the synthesis and calcification of the gastrolith itself. Accordingly, it is the aim of the present paper to deal with histological changes which are associated with the formation of non-calcified skeletal components of the gastrolith discs and those involved in the synthesis and calcification of the gastrolith itself.

MATERIALS AND METHODS

Animals. Since the duration of each stage of the molting cycle, the length of the intermolt periods, and frequency of molts for each size group have not been established for either field or laboratory *Orconectes virilis*, only males ranging in size from 40–49 mm. carapace length and molting during July and August were used in these studies. All animals were previously collected from the Cambridge Reservoir and maintained in the laboratory in concrete tanks containing rocks and soapstone slabs placed over the bottom to provide secluded areas for crayfish retreat and basking. A gently flowing stream of water, ranging in temperature from 21.8–25.0° C., was maintained at a level of approximately 2.5 inches in the tanks.

Stages of the molting cycle. Stages of the molting cycle were determined according to Drach (1939, 1944). This method is based primarily on morphological features of the exoskeleton, and clearly delimits four major stages—A, B, C, D—each of these being divisible into a number of substages. *Postmolt*, consisting of Stages A, B, early, and middle C, is characterized by progressive hardening of the pre-exuvial layers; formation, progressive thickening and hardening of the endocuticle; the beginning of feeding; and formation and progressive thickening of the membranous layer. The *intermolt condition* (Stage C₄) is characterized by the completion of all components of the exoskeleton—the epicuticle, exocuticle, endocuticle, and membranous layer. At this stage, one of comparative “rest” or “stability,” the completed membranous layer with the adhering epidermis can be stripped from the remaining portion of the exoskeleton. *Premolt*, consisting of early, middle, and late Stage D, is characterized by a series of integumentary transformations which occur preparatory for the ensuing molt. The major portions of the old exoskeleton, both organic matrix and mineral salts, are resorbed; the new pre-exuvial layers, consisting of the epicuticle and exocuticle, are deposited under the old; the animals cease to feed; and the termination of the period is marked by the molt. Stage D₀ is a new stage, previously referred to by Drach in connection with the shrimp molting cycle (Comments at Harvard, 1957). Stage D₀ in the crayfish is a stage in the molting cycle which cannot be distinguished from Stage C₄ by external features of the exoskeleton. The membranous layer with the adhering epidermis can be stripped from the exoskeleton in both stages but in Stage D₀, gastrolith deposition has begun, the gastroliths in this case being thin plate-like structures.

Histological methods. Three to five animals were killed for each specific stage of the molting cycle. The gastrolith discs were quickly removed, placed on a glass slide and straightened in a drop of fixative before they were transferred to bottles containing larger volumes of fixative. Although the author has used various fixatives for general histological studies of crustacean material (1951, 1955, 1957),

Bouin's fixative containing 1% calcium chloride (anhydrous) was used in these studies and has proved to be the best fixative. Fixation was carried out for at least a 24-hour period, followed by washing in 70% ethyl alcohol for the same period, dehydration in 80%, 95%, 97% ethyl alcohol for 30 minutes each, two changes of 100% ethyl alcohol for 15 minutes each and infiltration according to Peterfi's Celloidin-paraffin Method (Pantin, 1948). Sections were cut at 6 μ , deparaffinized, coated with 0.5% celloidin for one minute, allowed to dry and stained with Mallory's triple stain and Ehrlich's haematoxylin and eosin for general histological studies.

OBSERVATIONS AND DISCUSSION

The intermolt condition—Stage C₄

Intermolt is marked not only by the completion of the calcified skeletal components and the membranous layer of the rigid exoskeleton, but by the completion of the non-calcified skeletal components of the gastrolith discs. The epicuticle of

CHANGES IN THE GASTROLITH DISC DURING THE MOLTING CYCLE

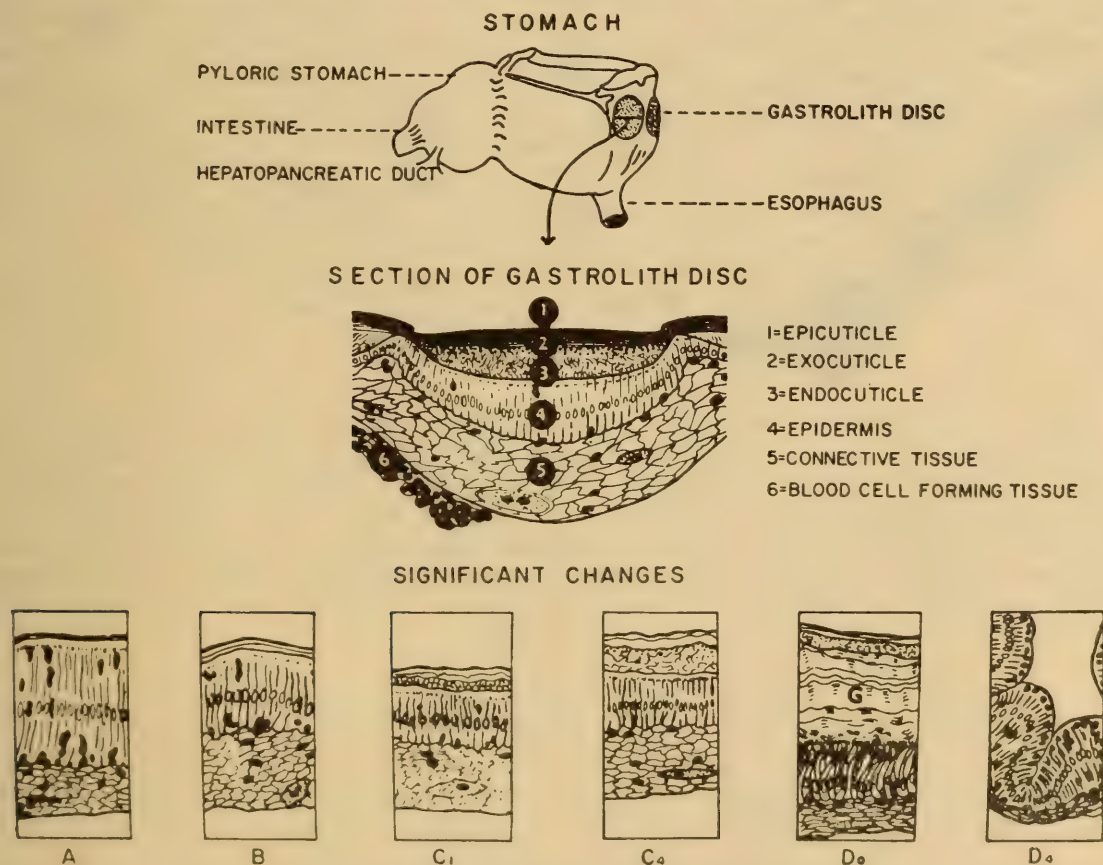


FIGURE 1. Diagram showing the histological changes which occur in the gastrolith disc during significant stages of the molting cycle. Enlarged section of the gastrolith disc is from an intermolt animal (Stage C₄), in which all skeletal components of the disc are completed, more clearly shown in Figure 2. Note significant changes in the development of cuticular components, epidermal height, connective tissue thickness, abundance of reserve cells (represented as black oval bodies). In Stage D₀, note gastrolith (G) forming between epidermis and cuticular components of the disc, and in Stage D₄, note hypertrophy and folding of the epidermis after it has retracted from the fully formed gastrolith and undergone a spurt of growth.

these latter components, adjacent to the lumen of the stomach, constitutes one surface of each gastrolith disc, while the basement membrane of the sub-epidermal connective tissue, adjacent to the hemocoel, constitutes the other surface of each disc (Figs. 1, 2, 4). Although the gastrolith discs, a few millimeters in diameter, are modified portions of the stomach wall, they can be distinguished from the remainder of the stomach wall in Stage C_4 by distinct differences in the structure and composition of their cuticular components and by the nature of their epidermis.

The completed skeletal components of the gastrolith discs of an intermolt animal show three major differentiated layers in contrast to the four observed in

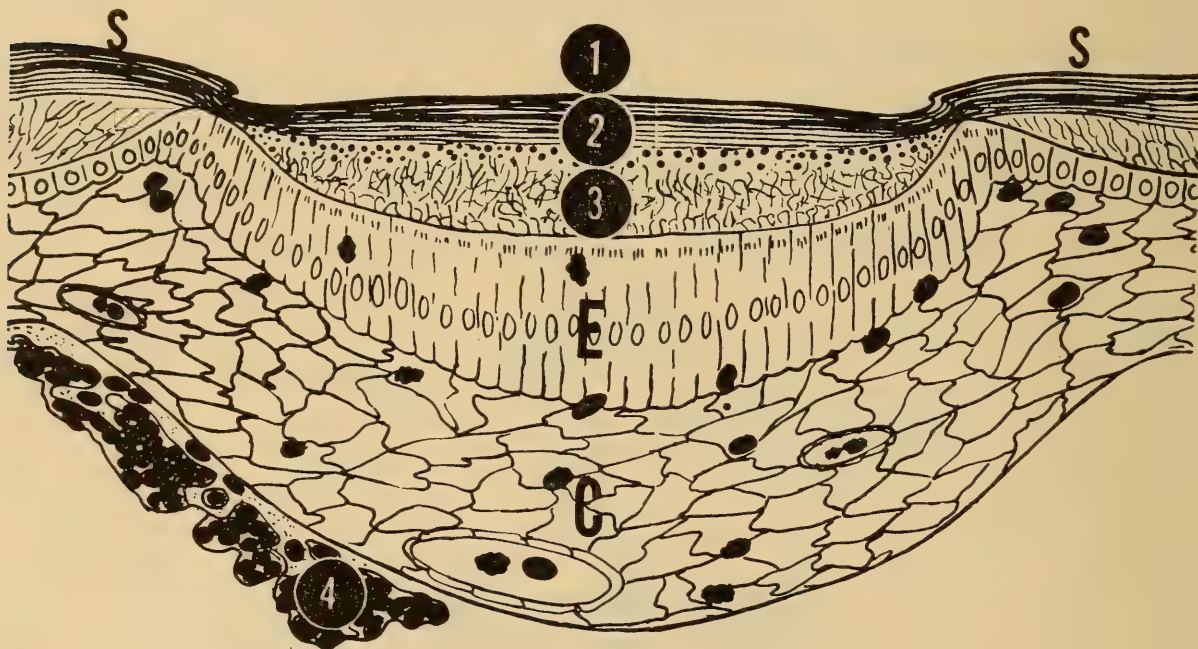


FIGURE 2. Enlarged diagram of the gastrolith disc of an intermolt animal (Stage C_4). Note the three differentiated skeletal layers—(1) epicuticle, (2) laminated exocuticle, (3) endocuticle with characteristic, chemically complex granules which mark the gastrolith disc histologically from the ordinary stomach lining (S). Note the tissue complex of gastrolith disc, which consists of a single layer of columnar epidermal cells (E), the sub-epidermal connective tissue (C), composed of cells of Leydig, and the transient reserve cells (represented as black oval bodies). Also note the blood cell-forming gland (4) and blood vessels in the connective tissue.

the more rigid branchial exoskeleton. Beginning with the lining of the stomach (Figs. 1, 2, 4), these are:

1. *The epicuticle.* This is a very thin non-calcified component, approximately $3-4\ \mu$ in thickness and composed of a glyco-lipoprotein complex (Travis, in preparation). It is neither laminated nor crossed by pore canals or tegumental ducts. Elaboration and hardening of the epicuticle begins before molt (Stage D_4) and is completed by Stage C_1 . This epicuticle differs from that of the branchial exoskeleton in being thinner and not being calcified or crossed by tegumental ducts. It stains red with Mallory's.

2. *The exocuticle.* This is a laminated component of approximately $8-12\ \mu$ in thickness and is also composed of a glyco-lipoprotein complex (Travis, in prepara-

tion). Deposition does not begin until late Stage A and is completed in Stage B. In contrast to the exocuticle of the branchial skeleton the gastrolith disc exocuticle is thinner, non-calcified, lacks pore canals and tegumental ducts, and is formed during a different stage of the molting cycle. The outer zone stains light blue with Mallory's while the inner stains dark blue, with a reddish haze at the junction of the exo- and endocuticle.

3. *The endocuticle.* This is the thickest of the three layers. It reaches a maximum thickness of 15–25 μ in Stage C₄ and stains very light blue with Mallory's. This layer differs from the same layer of the branchial exoskeleton in that it is neither laminated nor crossed by pore canals or tegumental ducts. The endocuticle begins to be deposited in late Stage B and is completed by Stage C₄. It appears as a loose fibrous meshwork and is marked by the presence of round structureless granules (Figs. 1, 2, 4), which also stain red with Mallory's. These granules reach a size of 2.5–3.0 μ , the largest being located immediately under the exocuticle. These are chemically complex granules which contain a glycoprotein complex, a mixed lipid complex, glycogen at certain stages of the molting cycle, and calcium (Travis, in preparation). Such granules are not observed in the endocuticle of the ordinary foregut lining.

The tissue underlying the skeletal components is composed of a single layer of columnar epidermal cells which lie in immediate contact with the endocuticle (Figs. 1, 2, 4). These columnar epidermal cells are approximately 40 μ in height with nuclei of about $5 \times 13 \mu$. The epidermal layer of the remaining portion of the stomach is a layer of cuboidal or low columnar cells.

The sub-epidermal connective tissue is very similar to that observed in the branchial integument. It is predominantly composed of the loose "spongy" type of connective tissue cells known as "cells of Leydig" (Figs. 1, 2, 4). A number of transient cells, called "protein cells" by Cuénot (1891, 1893), blood corpuscles by Hardy (1892), "reserve cells" (Travis, 1951, 1955, 1957), "lipoprotein cells" (Sewell, 1955), are also evident during the intermolt condition. These transient or reserve cells contain both acidophilic and basophilic granules when stained with haematoxylin and eosin and are usually fuchsinophilic with Mallory's. The granules vary in size, as observed with both the light and electron microscope (Travis and Chapman, II), from less than one micron to around four microns, and have the same histochemical composition as observed in the granules of the endocuticle (Travis, in preparation). The fine-structure studies indicate that the mature reserve cells contain large refractile granules which show variable densities and double membranes, while the immature cells are smaller, may contain clearer non-refractile cytoplasm and smaller granules of variable densities. These cells, among other functions, appear to be intimately involved in transport and release of reserves for synthesis and elaboration of skeletal elements (a more detailed discussion of these cells will be given in a subsequent paper of this series). In addition to these cellular elements, the sub-epidermal connective tissue is well penetrated by branches of the antennary arteries and blood sinuses. Also present in the connective tissue is a conspicuous blood cell-forming gland (Figs. 1, 2), in which stages of amoebocyte or reserve cell development may be seen. This blood cell-forming gland has also been observed by Małaczyńska-Śuchcitz and Hryniewiecka (1958). Delimiting the connective tissue from the hemocoel is a basement membrane.

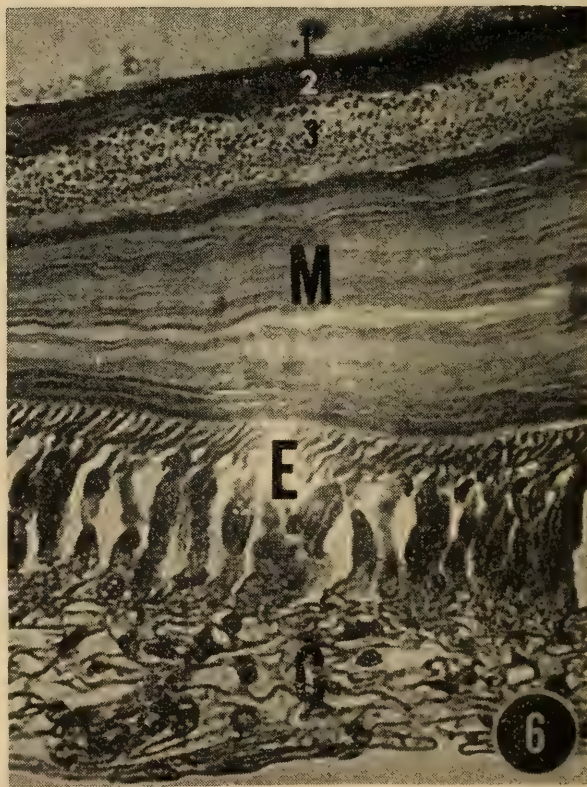
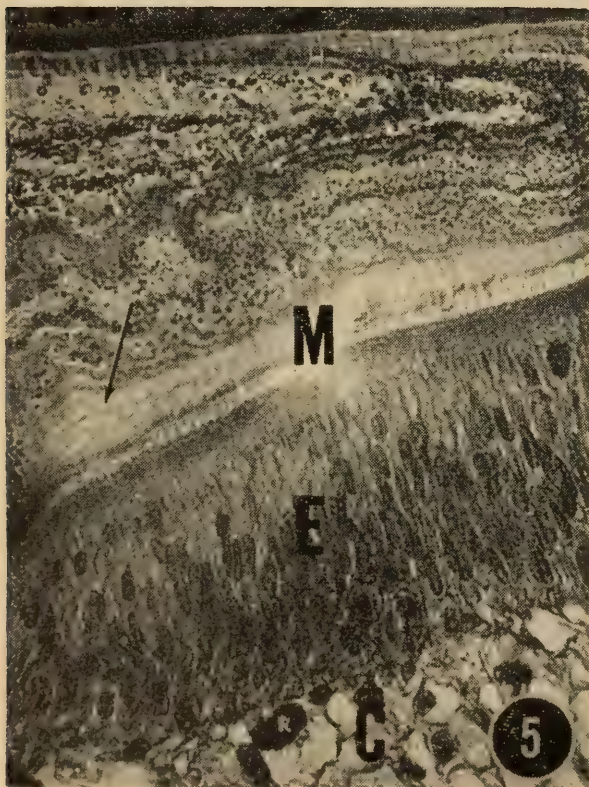
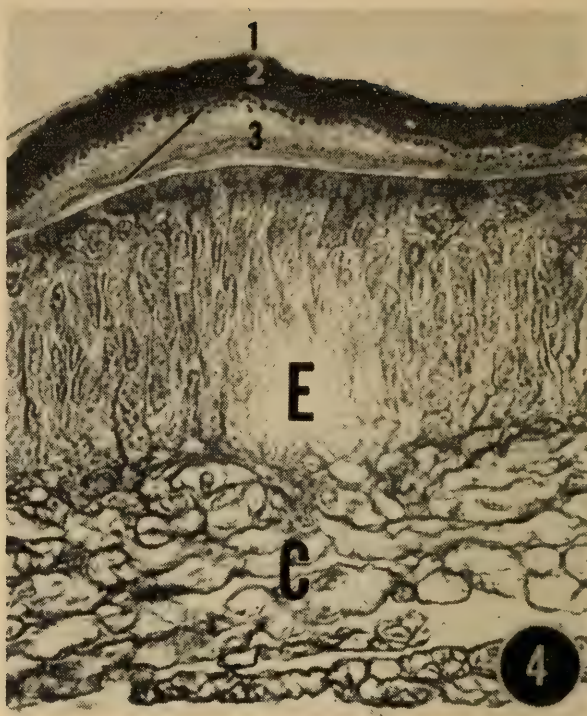
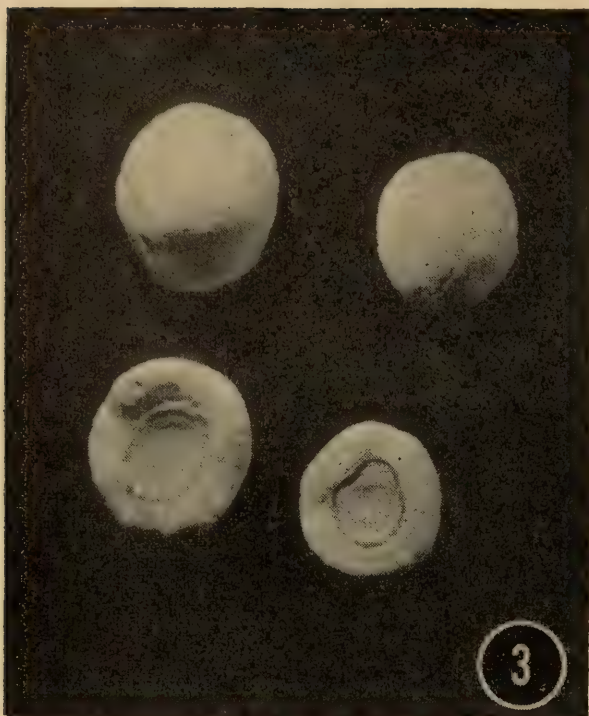


FIGURE 3. Two pairs of gastroliths removed from the gastrolith pouches. The exposed convex surface of the upper pair would lie *in situ* in immediate contact with the epidermis. The concave surface of the lower pair would lie in immediate contact with the stomach lining. 3 $\times$.

FIGURE 4. Section of the gastrolith disc skeletal-tissue complex (Stage C₄). The epicuticle (1), the exocuticle (2), the endocuticle (3) containing its characteristic chemically complex granules (arrow), the epidermis (E) and the connective tissue (C). 1800 $\times$.

Premolt-Stage D₀ through D₄

During this period of the molting cycle, marked histological changes, associated with gastrolith deposition, occur in the gastrolith disc tissues. In Stage D₀, the epidermis (with cells of about 75 μ in height and bearing nuclei of around $5 \times 12 \mu$) becomes somewhat invaginated. In section, this is evident at the very edge of the gastrolith disc (Fig. 5). These active epidermal cells shortly become branched and attenuated at their apical ends and many intercellular spaces are then apparent between the cells (Figs. 1, 6). Their attenuated apices lie in immediate contact with the forming matrix of the gastrolith. At the ultrastructure level, it is evident that the attenuated apices observed with the light microscope also bear innumerable microvillar equivalents (Travis and Chapman, III). From the microvillar equivalents both organic and mineral material are secreted, some microvillar equivalents secreting granular material into the forming amorphous matrix while others secrete distinctly beaded-fibrous material. The secreted organic components, not observable with the light microscope, undergo polymerization to form the fibrous lamellae (Travis and Chapman, II, III) clearly observed in the matrix of the gastrolith with the light microscope (Figs. 6, 7, 8). Evidence at the ultrastructure level also reveals that calcium transport and deposition appear to occur by two different means (Travis and Chapman, III). The granular and beaded-fibrous components secreted from the microvillar equivalents undergo a change. The former gives rise almost immediately to small crystals of calcite ranging in size from 440–1060 Å, while the beaded components of the latter eventually give rise to the same size crystals, presumably of calcite. Crystal formation from the beaded-fibrous components occurs simultaneously with polymerization changes associated with the formation of the fibrous lamellae of the matrix (Travis and Chapman, III). These small crystals, which form in the matrix of the young gastrolith (Stage D₀) and which are not observed with the light microscope, grow to a size of 7 μ or more in diameter and are clearly observed in ground sections of the completed gastrolith (Figs. 12, 13). These ground sections of the completed gastrolith (Stage D₄) also reveal that there is a shift in the mode of crystal deposition as the youngest and last layers of the fully completed gastrolith are deposited. In this case, crystals are deposited in such a fashion that rows of crystal prisms or rods (Fig. 10) are laid down parallel to the longitudinal axis of the epidermal cells and perpendicular to the parallel lamellae of the gastrolith (Fig. 9). The thickness of the younger layers of the gastrolith is constituted of these crystal prisms or rods, with one crystal stacked vertically above another. This thickness is 490–500 μ as compared to the older portions of the gastrolith (Figs. 9, 11, 12, 13), which constitute a thick-

FIGURE 5. Section through the extreme edge of the developing gastrolith (Stage D₀). Note that the developing matrix (M) is filled with granules similar in appearance and chemical composition to those observed in the completed endocuticle. Also note beginning appearance of fibrous lamellae (arrow). Epidermis is denoted by (E), connective tissue by (C) and reserve cells (R). 1800 $\times$.

FIGURE 6. Section through a more central portion of the developing gastrolith (Stage D₀). Note the distinct fibrous lamellae of the gastrolith matrix (M) which result from post-secretion polymerization changes, the attenuated apices of the epidermal cells (E) and the intercellular spaces between the cells, and the connective tissue (C). Also note the three differentiated skeletal layers of the gastrolith disc—(1) epicuticle, (2) exocuticle, (3) endocuticle with characteristic granules. 1800 $\times$.

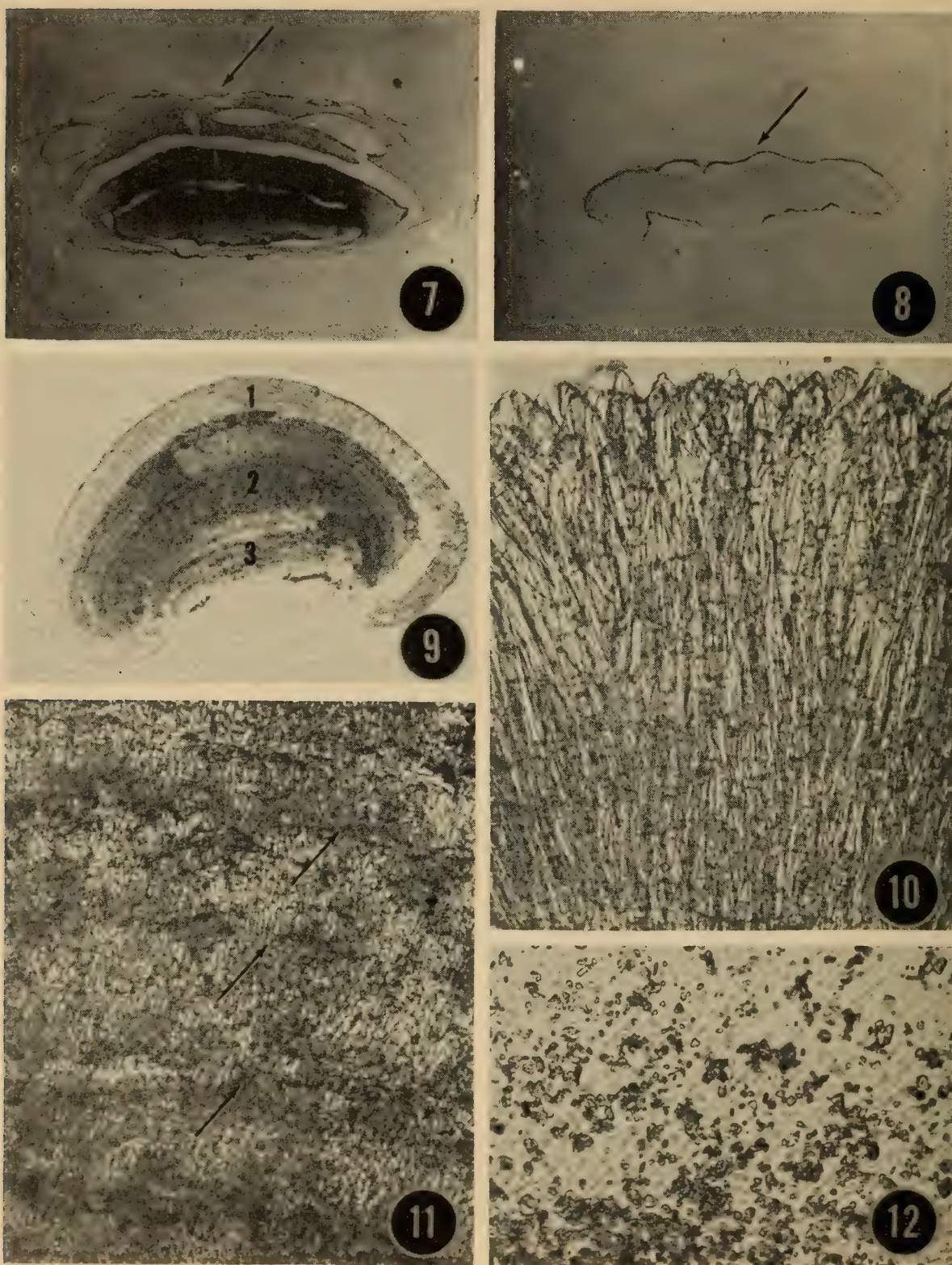


FIGURE 7. A section through the organic matrix of a completed gastrolith (Stage D₄) showing its fibrous lamellate nature. Fixed and decalcified in ordinary Bouin's and stained with toluidine blue. The more convex surface (arrow) would lie *in situ* in immediate contact with the epidermis while the opposite surface would lie in contact with the stomach lining. 16 ×.

FIGURE 8. A similar section through the organic matrix of the early developing gastrolith (Stage D₀), showing its fine fibrous lamellate nature. Arrow indicates surface (younger layers) which would lie *in situ* in contact with the epidermis. 21 ×.

ness of 2500–3000 μ and which show lamellae and crystals oriented at right angles to the prisms. Preliminary x-ray diffraction studies indicate that the main inorganic crystals in both the youngest and oldest layers of the gastrolith are calcium carbonate deposited as calcite.

The sub-epidermal connective tissue becomes greatly compressed or stretched during this stage of the molting cycle. This is largely due to the size of the developing gastrolith which is plate-like in nature (Stage D_0) and has attained a thickness at this time of about 300 μ . By middle Stage D, the gastrolith has increased to a thickness of approximately 1 mm. and by the end of D_4 , a thickness of approximately 3–4 mm. is achieved in animals of 40–49 mm. carapace length. Reserve cells remain numerous in the connective tissue and epidermis during the entire pre-molt period, being heavily concentrated in the apices of the epidermal cells, and achieve their greatest abundance in late Stage D (D_4). During this latter stage the epidermis has completed the deposition of the gastrolith and retracts from it, undergoes rapid growth, accompanied by much folding, and achieves its greatest height (242 μ) and nuclear size of $9 \times 14 \mu$ (Fig. 14). It now begins to elaborate a new epicuticle which is the only skeletal component of the gastrolith disc deposited before molt. In areas of the stomach wall on each side of the gastrolith discs both pre-exuvial layers, epi- and exocuticle, have been deposited. When synthesis of the gastrolith and new epicuticle are achieved by the gastrolith disc epidermis, correlated with the many tasks the epidermis performs in other skeletal areas, molting ensues and the old stomach lining and the completed gastroliths (Fig. 3) are released into the stomach.

Postmolt-Stage A, B and C

Stage A. The tissue complex of the gastrolith disc remains greatly hypertrophied. The epidermis, in contrast to the folded appearance in Stage D_4 , is a flat-surfaced tissue, composed of rows of columnar cells, which maintain a height of approximately 182 μ and an average nuclear size the same as that of a D_4 animal (Fig. 15). The reserve cells remain abundant in both the epidermis and connective tissue, with the greatest number being observed in the apices of the epidermal cells. The only skeletal component observed in the gastrolith disc early in Stage A

FIGURE 9. A ground section of a fully developed gastrolith (Stage D_4). The youngest portion (1) would be in contact with the epidermis, and consists of crystal prisms or rods, also indicated in Figure 10, which are laid down parallel to the longitudinal axis of the epidermal cells and perpendicular to the parallel lamellae of the gastrolith. Region (2), also represented in Figure 11, is an older portion of the gastrolith showing the parallel lamellae with crystals oriented at right angles to the prisms of region (1). Region (3) represents the oldest layers of the gastrolith, originally corresponding to Stage D_0 , and would lie in contact with the skeletal layers of the gastrolith disc which constitute part of the stomach lining, also represented in Figures 12 and 13. 8.5 $\times$.

FIGURE 10. Enlarged portion of region (1) from a ground section of a completed gastrolith, depicted in Figure 9. Note crystal prisms or rods of calcite. 400 $\times$.

FIGURE 11. Enlarged portion of region (2) from a ground section of a completed gastrolith depicted in Figure 9. Note that crystals of calcite in the lamellae (arrows) lie at right angles to the crystal prisms of region (1). 400 $\times$.

FIGURES 12, 13. Enlarged portions of region (2) from a ground section of a completed gastrolith depicted in Figure 9. Note same arrangement of crystals of calcite—7–70 μ in diameter—as observed in Figure 11. 400 $\times$.

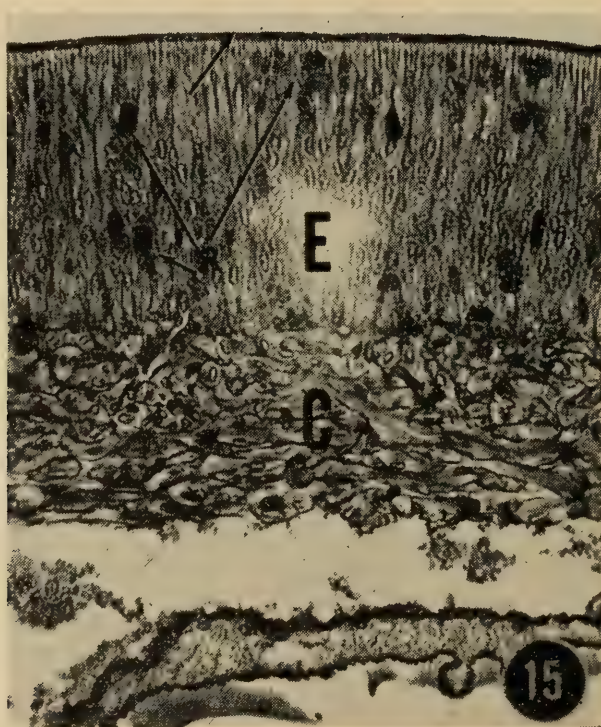
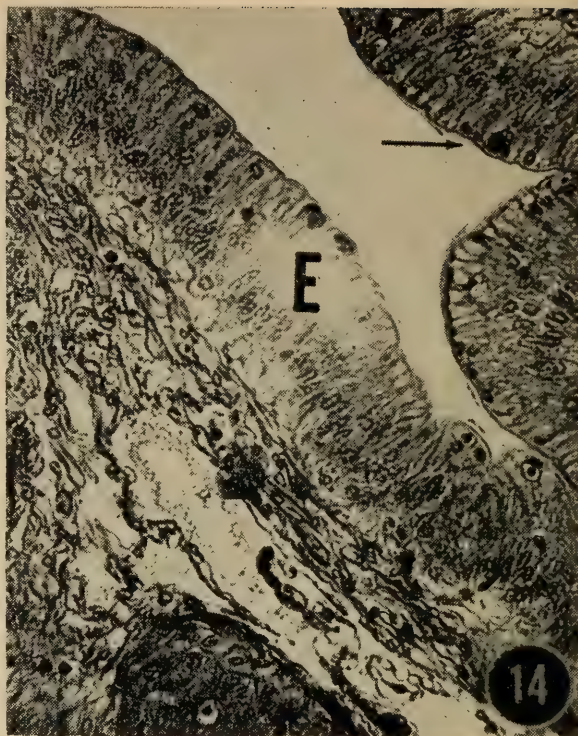
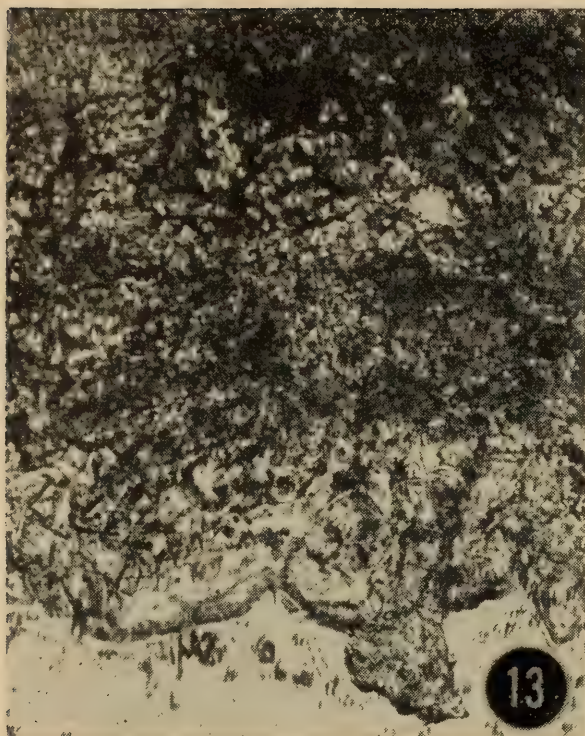


FIGURE 14. Section of gastrolith disc showing greatly hypertrophied and folded epidermis (E), following retraction and growth of this tissue after gastrolith deposition is complete (Stage D₄). Note beginning of new epicuticle formation (arrow) and numerous dark staining reserve cells in the apices of the epidermal cells. 900 $\times$.

FIGURE 15. Section of gastrolith disc immediately following molt (Stage A). Note the greatly hypertrophied flat-surfaced epidermal cells (E), the epicuticle (arrow), the only skeletal component evident in early Stage A, and the numerous dark staining reserve cells (arrow-R), and connective tissue (C). 900 $\times$.

is the epicuticle, partly formed and hardened previous to molt and having a thickness of only 1.6–2.0 μ . Curiously enough, the exocuticle does not apparently begin to be deposited until late Stage A and is completed in Stage B, achieving a thickness of approximately 8–12 μ . In the areas of the stomach wall on each side of the gastrolith discs, the exocuticle is completed and endocuticle formation has already begun.

Stage B. This stage of gastrolith disc formation is marked by the completion of the synthesis and elaboration of the exocuticle. It is both fibrous and laminated in nature. This latter aspect is not clearly evident at the microscopic level, but is distinctly apparent in electron micrographs (Travis and Chapman, II). The synthesis and elaboration of the endocuticle also begins during the latter part of this stage but is not completed until late Stage C.

There is a slight decrease in epidermal cell height (112 μ) and nuclear size ($5.1 \times 11.5 \mu$) and an increase in thickness of the connective tissue when compared to the situation observed in Stage A. During this stage the reserve cells are still quite abundant.

Stage C. Early Stage C (C_1) and middle Stage C (C_2) are marked by the reduction in epidermal height (about 80 μ), a slight increase in nuclear size ($5 \times 13 \mu$), an increase in thickness of the sub-epidermal connective tissue, a gradual reduction in the number of reserve cells, and the continued synthesis of the endocuticle, previously described under the intermolt condition. It is during Stage C_1 that the interesting granules, observed in the gastrolith disc endocuticle, become evident at the ultrastructure level, although not becoming distinctly apparent at the light microscope level until Stage C_4 . In the tissue complex there is little difference between the early and middle Stage C condition and that observed in the intermolt animal. At the end of Stage C when synthesis of the gastrolith disc skeletal components is complete, the animal has again reached an "intermolt" condition (C_4) from which the entire cycle repeats itself. Further information on the development of the non-calcified skeletal components of the gastrolith disc and of the calcified gastrolith itself will appear in subsequent papers of this series.

SUMMARY

1. The gastrolith discs of the fresh-water crayfish, *Orconectes virilis* Hagen, are located in the anterior lateral walls of the cardiac stomach and are themselves modified portions of the stomach wall.

2. Histologically, the cuticular surface of the completed gastrolith disc (Stage C_4), which forms part of the lining of the stomach, is composed of three differentiated layers: a thin non-calcified epicuticle, formed before molt; a thicker non-calcified exocuticle, laminated in nature, but neither crossed by pore canals, nor tegumental ducts, formed following molt and completed during Stage B; and the endocuticle, the thickest of the three layers, characterized by neither being laminated nor crossed by pore canals and possessing round granules without structure, embedded in a loose fibrous meshwork. The largest of these granules are located in the outer layers of the endocuticle very close to the exocuticle. The tissue complex of the completed gastrolith disc is composed of a single layer of columnar epidermal cells, a loose "spongy" type of sub-epidermal connective tissue, composed of cells of Leydig and a number of the transient reserve cells which are also observed in the

epidermis. The tissue complex is well supplied by branches of the antennary arteries and has a large blood-cell-forming gland.

3. Marked histological changes are observed during Stage D when premolting processes, associated with gastrolith deposition, are set into motion. The epidermis increases greatly in height, invaginates, and begins to elaborate the matrix of the gastrolith which is filled at its lateral edges with granules like those observed in the C_4 endocuticle. At this same time the active epidermal cells take on a branched attenuated appearance at their apical ends, and many intercellular spaces become apparent between the cells. From Stage D_0 through most of Stage D_4 continued synthesis, elaboration, and calcification of the gastrolith matrix occur, its thickness increasing from around $300\ \mu$ (D_0) to 3–4 mm. (D_4). Concomitant with these changes is an increase in the number of reserve cells in both epidermis and connective tissue and a stretching, and thus compression, of the sub-epidermal connective tissues, the latter process being correlated with the increasing size of the forming gastrolith. Near the end of Stage D (D_4), the epidermis has completed its task of depositing the gastrolith, retracts from it, undergoes rapid growth accompanied by much folding and achieves its greatest height. It then begins to elaborate the epicuticle, the only pre-exuvial skeletal component deposited in this site. When this task is achieved by the epidermis, correlated with the many tasks it performs in other skeletal areas, molting ensues and the old stomach lining and the formed gastroliths are released into the stomach.

4. Postmolt histological changes in the gastrolith disc are associated with continued synthesis and elaboration of cuticular components. The "intermolt condition" is achieved in Stage C_4 , when these synthetic tasks are complete, and from this stage the entire histological cycle repeats itself.

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EFFECT OF TEMPERATURE AND SALINITY ON HEAT TOLERANCE IN TWO GRAPSOID CRABS, HEMIGRAPSUS NUDUS AND HEMIGRAPSUS OREGONENSIS¹

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Temperature tolerance as an experimental criterion for the demonstration of physiological change has found many uses and has been reported in various ways throughout the literature. Most studies on temperature tolerance have been done on fish, with relatively few experiments on invertebrates. In many cases the lethal point was determined by the temperature at which death occurred when the animal was subjected to slowly increasing temperatures (Huntsman and Sparks, 1924; Gowanloch and Hayes, 1926). As various authors raised the temperature at differing rates, the results could not be compared adequately because the time over which the temperature was increased would be expected to have a marked effect on the final lethal point. Experiments in which animals were maintained at constant temperatures for a period of time provided a more accurate determination of the lethal temperature as well as allowing comparisons among the species. In this type of experiment, either time to death or per cent survival at intervals was noted (Fry, Brett and Clawson, 1942; Brett, 1952; Spoor, 1955).

Acclimation to low and high temperatures has been demonstrated in many animals on the basis of laboratory acclimation, season, microgeography and latitude. In this discussion, "acclimation" includes all types of "demonstrable compensatory change" (Bullock, 1955). Acclimation has been demonstrated by a higher rate function at any intermediate temperature when low temperature acclimated animals are compared with high acclimated ones. Dehnelt and Segal (1956) in laboratory studies on the American cockroach, *Periplaneta americana*, found a higher rate of oxygen consumption in equal-weight nymphs and adults after acclimation to 10° C. when compared with 26° C. acclimated animals. Respiration was measured at 20° C. Previously, the culture had been maintained for at least three generations at a constant temperature, 27° C. Edwards and Irving (1943) showed seasonal acclimation in the sand crab, *Emerita talpoida*. At all experimental temperatures below 20° C., oxygen consumption of animals in summer was less than that in winter. Ohsawa and Tsukuda (1956) found seasonal acclimation of response to temperature (exuding of the body) in the periwinkle, *Nodilittorina granulatis*. Microgeographical acclimation has been demonstrated in the limpet, *Acmaea limatula*. Samples taken from lower intertidal levels had a higher rate of heart beat than those from higher levels (Segal, 1956).

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Mayer (1914) has shown that at different latitudes there was a similar rate function within a species at the environmental temperatures of the individuals, even though these temperatures were different. The rate of pulsations of the bell of *Aurelia aurita* was similar in animals off Nova Scotia and off Florida with a temperature difference of 15° C. Scholander, Flagg, Walters and Irving (1953) compared many poikilotherms, mostly interspecific but closely related species, from arctic and tropic regions to find the general consistency of acclimation when rates of oxygen consumption were investigated. Dehnel (1955) demonstrated a higher rate of growth in gastropods from high latitudes when compared with low latitude populations.

Another facet of acclimation is its effect on the temperature tolerance of a species. A previous temperature history at the upper levels of the physiological temperature range is known to raise the thermal resistance. Conversely, low temperature acclimation will decrease the tolerance to high temperatures. The rates of gain and loss of heat tolerance appeared to show a consistent trend in diverse groups of animals. In all cases, gain of heat tolerance was much more rapid than its loss. Brett (1946) demonstrated that the goldfish, *Carassius auratus*, required a total of thirty days to acclimate to 28° C. when brought from 4° C. in 8° C. steps, but that acclimation occurred at different rates. Twenty days were required from 4° C. to 12° C., but only three days from 20° C. to 28° C. Loss of heat tolerance in the crayfish, *Orconectes rusticus*, was not completed by the end of the sixteenth day when the crayfish were transferred from 22°–23° C. to 4° C. The heat tolerance lost after thirteen days at 4° C. was regained in about 24 hours (Spoor, 1955). Ohsawa (1956a) using the periwinkle *Nodilittorina granularis*, stated (p. 206), "The acclimatization to higher temperatures in the winter snails is established more readily than the acclimatization to lower temperatures in the summer animals."

The effects of salinity changes on temperature tolerance have been studied less extensively. Broekema (1941) demonstrated that the temperature and salinity relations were interdependent in the shrimp *Crangon crangon* when survival in various combinations of the two was tested; a low salinity was endured better when the temperature was high. Wikgren (1953) showed that there was almost a continuous loss of ions from the crayfish, *Potamobius fluviatilis*, as the temperature dropped below 2° C. from about 18° C. when animals were transferred from tap to distilled water. There was apparently more efficient osmoregulation, normal loss of ions, at higher temperatures (above 2°–3° C.). The lobster, *Homarus americanus*, was shown to have a higher lethal point when both salinity and temperature were high during the period of acclimation. A decrease in salinity with the acclimation temperature held constant resulted in a lowering of thermal resistance (McLeese, 1956).

The two species of shore crabs studied in this investigation, *Hemigrapsus nudus* (Dana) and *H. oregonensis* (Dana), are subjected to wide ranges of temperature and salinity conditions in their natural environment. Experimental parameters of temperature and salinity were based on average seasonal conditions, to determine if these seasonal changes, summer and winter, cause a resulting change in the upper temperature tolerance of the two species. Also, animals were acclimated to various combinations of temperature and salinity, to ascertain any resulting changes in

the temperature tolerance within either season. Because of the morphological similarity and like habitat in this area of these two species, it was hoped that physiological differences, in this case resistance to high temperatures, could be demonstrated. This was found to be the case. Additionally, effects of size, crowding, sex and moulting on the tolerance were noted.

MATERIAL AND METHODS

The experimental animals, *H. nudus* and *H. oregonensis*, were collected from Spanish Bank Beach on the south shore of Burrard Inlet. The Fraser River flowing into Georgia Strait near the collecting beach is the main cause of the recorded fluctuations in salinity occurring in this area. The beach has a gradual slope and changes its contour from sand to rocks and again to sand. The lower sandy area is exposed only on the lower tides. The crabs are found in the rocky intermediate area, the main population of *H. nudus* distributed higher than that of *H. oregonensis*. Individuals of both species, however, can be found almost from highest occurrence to lowest.

The salinity is quite uniform throughout the winter, ranging from about 70 per cent to 80 per cent sea water (based on 100 per cent sea water as 31.88‰). In the spring, with the greater influx of fresh water from the Fraser River, the salinity begins to drop and has been recorded as low as 10 per cent in the summer months. At the same time as the salinity is decreasing, temperature increases, and the relatively static summer state results where the average salinity is about 35 per cent. Daily fluctuations in the salinity also occur, as the incoming tide carries a less saline water along the shore in the collecting region. This muddier Fraser River water is distinct from the water of higher salinity by a visual demarcation several hundred yards from shore.

The two species of crabs are found under rocks where a suitable micro-environment is provided by a pool of water or a bed of mussel shells (*Mytilus edulis*). Both species are found also in damp sand under rocks, often partially buried. Both males and females were used in the temperature tolerance experiments when preliminary experiments showed no difference in the resistance of the sexes. Gravid females or individuals missing any appendages were discarded, as were any soft-shell crabs. A weight range was collected, about 0.2-gm. to 6.0-gm. animals, excluding only very large individuals. Crabs were transported to the laboratory in canvas buckets with damp sea-weed. The experiments reported here were conducted from 1957 to 1959, inclusive.

In this investigation, *holding experiments* refer to those in which the temperature tolerance of the crabs is determined without any previous laboratory acclimation. The animals were tested after at least 24 hours in the laboratory, and during that period they were held at temperature and salinity conditions which approximated those in the field. This time period allowed the gut to be cleared partially so that at the high test tolerance temperatures, deposition of faeces and urine did not foul the water. In *acclimation experiments*, on the other hand, animals were held at previously determined acclimation conditions which differed from environmental circumstances in at least one factor. In these experiments crabs were acclimated for at least one week in the laboratory. Preliminary experiments indi-

cated that acclimation was complete in less than seven days; the tolerance of the two species, therefore, was tested from seven to a maximum of twenty-one days in some experiments. Crabs were not fed at any time during the experiments, and were kept in darkness.

If the acclimation temperature differed greatly from the environmental temperature, the crabs were gradually warmed or cooled until the desired temperature was reached, usually a period of two hours. Plastic dishes containing slightly less than 4 liters of water were used for the experimental animals. Holding or acclimation temperatures were either 5°C . or 20°C ., $\pm 1^{\circ}\text{C}$., based on average winter and summer temperatures. The salinities were either 75 per cent or 35 per cent sea water, again average seasonal conditions. Hence, four experimental combinations were used. About thirty-five crabs were placed in each container. Animals were changed, usually once per 24 hours, to water of appropriate temperature and salinity over the acclimation or holding period.

Temperature tolerance tests were conducted by floating the plastic containers in water baths in which the experimental temperature did not vary more than $\pm 0.1^{\circ}\text{C}$. The appropriate salinity, corresponding to that used during the acclimation or holding period, was employed for each tolerance test. Five animals per dish (per 4 liters of water) were tested at 1°C . intervals, from 28°C . to 35°C . The five crabs tested at each temperature ranged in weight from small to large. At each temperature both males and females were tested. Crabs were brought to the test temperature from acclimation or holding temperatures over a period of two to four hours at which time the experiment was begun. Preliminary experiments indicated that more time was unnecessary as a more gradual warming period did not alter the results. The experiment was conducted for 24 hours; dead animals were removed at 12 hours and live ones were changed to clean water. All animals were dried with gauze and weighed to the nearest 0.1 gram. Animals were considered dead only when all movement ceased. Using this criterion as a death point, at no time did animals revive when returned to lower temperatures. Immediately prior to death, the sole motion visible was a slight pumping of water through the gill chambers. Crabs which moulted during the 24 hours of the experiment were discarded.

The relationship of weight and time to death was determined by checking at hourly intervals over the 24-hour period and removing and weighing any dead animals. There was a change of water at 12 hours as noted previously. One set of experiments was used to test the effect of different numbers of crabs per dish on the thermal resistance. All experimental conditions were as described in the foregoing except for the varying densities of animals at each test temperature.

Data were plotted on arithmetic paper as it was believed that this type of plot gave the most accurate representation of the temperature tolerance curve. Each point, indicating per cent survival at any given temperature, represented the average of a number of duplications (each five animals per dish) for those particular conditions. In no instance was this average determined from less than six replications resulting in a total of at least thirty animals; usually more were used. The total graph, then, was based on a minimum of one hundred-eighty animals. No attempt was made to derive a formula for the fitting of each curve; the points were merely joined. The 50 per cent survival temperature was based on the

point at which the survival curve crossed the line representing median survival of the animals. In the graph which represented the relationship between size and time to death, the regression line is eye-fitted.

An analysis of variance was done for the complete summer data mainly to determine if a species difference existed. The difference in tolerance between the species was less during the summer months, so that a significant difference here definitely indicated one for the complete data, both summer and winter. For individual comparisons such as the difference in tolerance between the species at a given temperature-salinity combination, the "Student's" *t*-distribution was used to compare the 50 per cent survival temperatures. Another method was the comparison of the total area under the curve for any two graphs, contrasting the total cumulative per cent survival. The statistical probabilities resulting from the *t* test using this method were approximately the same as when 50 per cent survival temperatures were compared. Since the latter is a value commonly found in the literature, this was chosen as a basis for comparison rather than the total cumulative survival value. In all cases where a statistically significant difference has been demonstrated using the *t* test, the level of significance is based upon the 0.05 probability point, although the probability in some instances is less than the 0.01 level.

RESULTS

SUMMER

Average conditions of salinity and temperature representing the summer months were 20° C. and 35 per cent sea water. Survival values obtained from summer animals with no laboratory acclimation, holding experiments, were considered as the base line curves. These curves were compared with heat tolerances of animals acclimated to 20° C., 75 per cent sea water; 5° C., 35 per cent sea water; and 5° C., 75 per cent sea water. Temperatures at which 50 per cent survival occurred for 12 and 24 hours were used as the basis for comparisons.

Holding Experiment: 20° C. and 35 per cent sea water

Results of the base line experiments showed that *H. oregonensis* was more resistant than *H. nudus* at both 12 and 24 hours. The temperature at which 50 per cent survival was found for *H. oregonensis* was 33.43° C. for 12 hours and 33.16° C. for 24 hours. For *H. nudus* the values were 32.95° C. for 12 hours and 32.50° C. for 24 hours (Fig. 1).

Acclimation Experiment: 20° C. and 75 per cent sea water

Both species showed a marked increase in tolerance after acclimation to 20° C. and 75 per cent sea water. This combination of temperature and salinity differed from the base line conditions only in the salinity, having been changed from 35 per cent to 75 per cent sea water. *H. oregonensis* again showed more tolerance than *H. nudus* with values of 34.50° C. for 12 hours and 34.38° C. for 24 hours, an increase of 1.07° C. and 1.22° C., respectively, over the base line values. The 50 per cent survival temperatures for *H. nudus* were 33.62° C. and 33.45° C. for

12 and 24 hours, an increase of 0.67°C . and 0.95°C . (Fig. 2). The increase found in both species was significantly different from the base line values, $P = .05$. It is important at this point to note that acclimation to the above conditions provided the most favourable background for withstanding the high test tolerance temperatures.

Acclimation Experiment: 5°C ., 35 per cent sea water

Low temperature and low salinity combined appeared to be slightly less favourable for survival during the acclimation period as the number of deaths in both species was greater than with any of the other combinations, but with fewer deaths among *H. nudus*. Results showed the tolerance to be much the same as the base line values, with no significant difference for 50 per cent survival, 33.28°C . and 33.11°C . for *H. oregonensis* and 33.38°C . and 32.64°C . for *H. nudus* for 12 and 24 hours (Fig. 3).

Acclimation Experiment: 5°C ., 75 per cent sea water

Acclimation to these conditions did not result in much change in the tolerance when compared with base line conditions. It is possible that the time allowed for acclimation was not of sufficient duration, since animals collected in the winter, when these conditions were found in the field, did show a lowering of the tolerance. However, no change was detected in the temperature at which 50 per cent survival occurred in the summer animals after seven days' acclimation compared with a total of thirteen days' acclimation. The temperatures for 50 per cent survival for *H. oregonensis* were 33.20°C . and 32.73°C . and for *H. nudus*, 33.05°C . and 32.58°C . for 12 and 24 hours (Fig. 4). These values did not have a significant difference from those found in the base line experiments. When these animals, however, were compared with animals acclimated to 20°C ., 75 per cent sea water (Fig. 2), a significant difference in tolerance was found, $P = .05$. Summer animals, then, when acclimated to both high and low temperatures with high salinity constant, demonstrated a reduction of thermal tolerance with the low temperature acclimation.

WINTER

The winter base line represented the heat tolerance of animals collected during the winter months. These were the winter holding experiments. Also, winter animals were acclimated to the three remaining combinations: 20°C ., 35 per cent sea water; 20°C ., 75 per cent sea water; and 5°C ., 35 per cent sea water.

Holding Experiment: 5°C ., 75 per cent sea water

The 50 per cent survival temperatures were 32.59°C . and 32.43°C . for *H. oregonensis* and 32.00°C . and 31.79°C . for *H. nudus* for 12 and 24 hours, respectively (Fig. 4). The difference between these winter values and those obtained for summer animals acclimated to these same conditions (winter) is significant, $P = .05$.

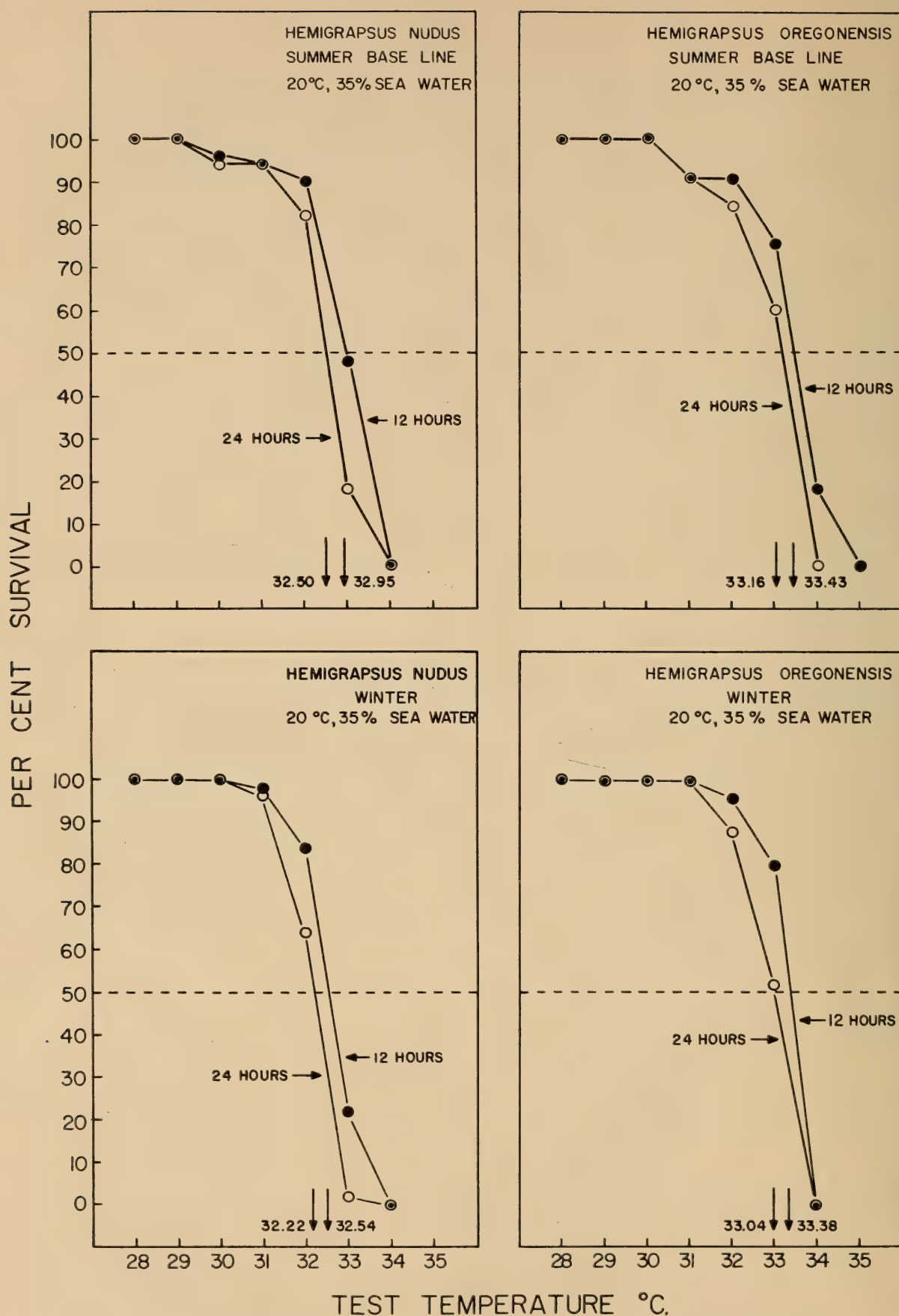


FIGURE 1. The influence of a previous history of 20° C., 35 per cent sea water on the 50 per cent survival values at 12 (●) and 24 (○) hours. Both species in summer and winter are shown. Each point represents the average survival of at least thirty animals.

PER CENT SURVIVAL.

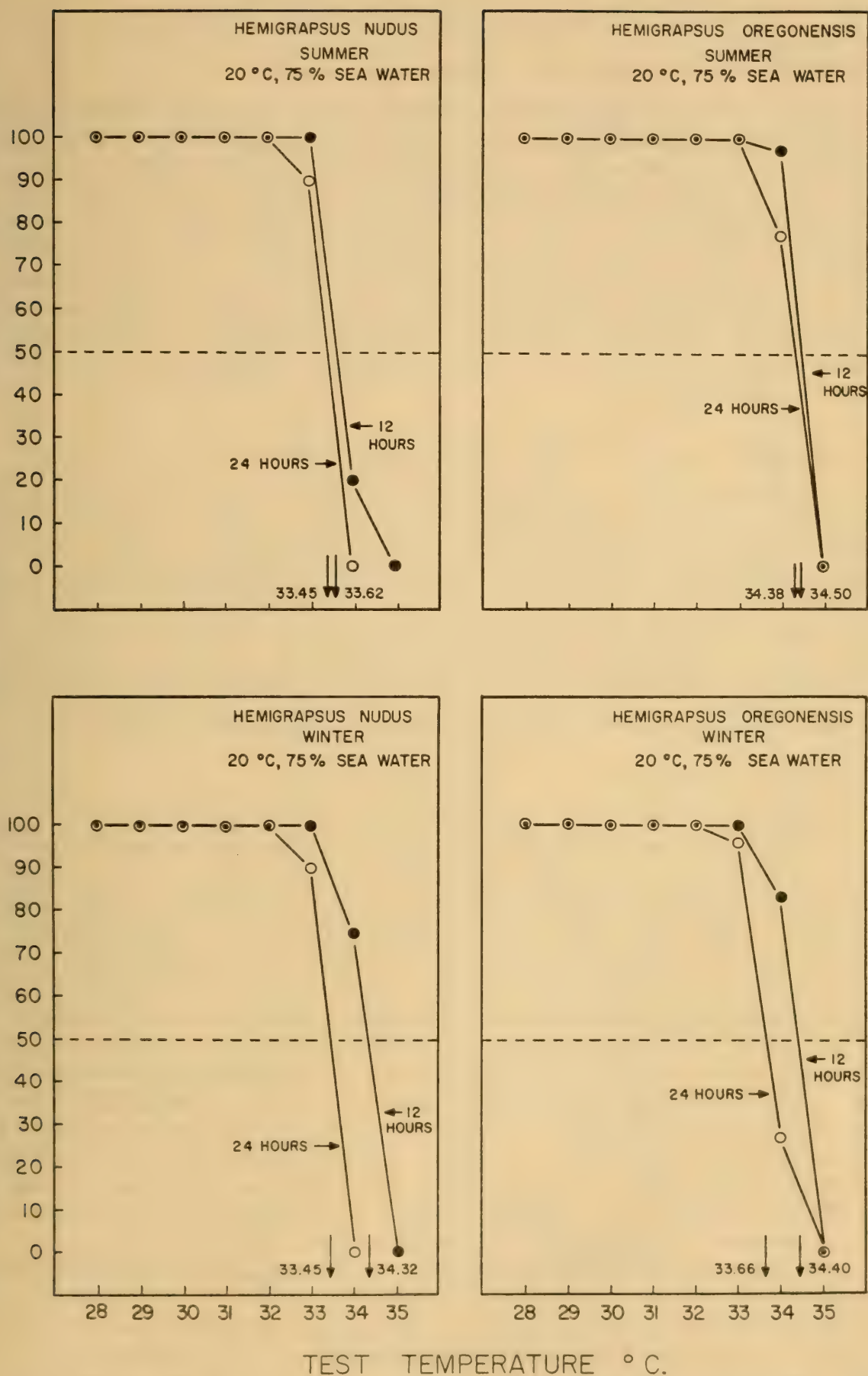


FIGURE 2. The influence of a previous history of 20° C., 75 per cent sea water on the 50 per cent survival values at 12 (●) and 24 (○) hours. Both species in summer and winter are shown. Each point represents the average survival of at least thirty animals.

Acclimation Experiment: 20° C., 35 per cent sea water

Crabs collected during the winter months and acclimated to the above conditions, which corresponded to the summer base line combination, were found to have survival values quite similar to those found in the summer months. The 12 and 24 hour 50 per cent survival temperatures were 33.38° C. and 33.04° C. for *H. oregonensis*, and 32.54° C. and 32.22° C. for *H. nudus*, the greatest difference being only 0.41° C. from the summer months (Fig. 1). This meant that in less than one week the winter animals were able to approach the degree of resistance found in the summer animals which may have had several months under these conditions in the field. There was a significant difference in the 50 per cent survival points between winter animals acclimated to summer base line conditions, and the winter base line.

Acclimation Experiment: 20° C., 75 per cent sea water

Acclimation to these conditions provided the most suitable environment to withstand the test tolerance temperatures; there was a marked increase in the temperature at which 50 per cent survival occurred, from the base line. Again, the animals acclimated in the winter approximated the tolerance found in summer animals acclimated to these same conditions, and were significantly different from the winter base line. In one instance, *H. nudus* at 12 hours, the tolerance was even greater. The 50 per cent survival values for *H. oregonensis* were 34.40° C. and 33.66° C., and were 34.32° C. and 33.45° C. for *H. nudus* for 12 and 24 hours (Fig. 2), an average increase of about 2° C. over the base line.

Acclimation Experiment: 5° C., 35 per cent sea water

Animals acclimated to the low salinity, low temperature combination showed the greatest difference, both from the summer counterpart and the winter base line. The 50 per cent survival temperatures for *H. oregonensis* were 30.18° C. and 30.13° C. for 12 and 24 hours, and for *H. nudus* 28.81° C. and 28.68° C. (Fig. 3). This was a drop of approximately 2.5° C. for *H. oregonensis* from the winter base line conditions and 3° C. from the survival temperatures obtained from summer animals acclimated to these same conditions. The drop in the survival temperatures for *H. nudus* was even greater, being about 3.0° C. and 4.5° C., respectively. When winter animals acclimated to both high and low temperatures with low salinity constant are compared, the low temperature acclimation results in a significantly different reduced tolerance ($P = .05$). During the acclimation period, the number of deaths was greater in both species than at any other temperature-salinity combination, a similar result to that found with the summer animals. Contrary to *H. nudus* being the slightly more resistant during the acclimation period in the summer animals, that species was less resistant than *H. oregonensis* in the experiments involving winter animals.

As seen from the results of the above experiments, the 50 per cent survival temperatures for both 12 and 24 hours were higher for *H. oregonensis* than for *H. nudus* in all except one case when the value for *H. oregonensis* was only 0.10° C. lower. Usually the 50 per cent survival temperature for *H. oregonensis* was higher by a much greater amount than this, being in some cases over one degree. Analysis

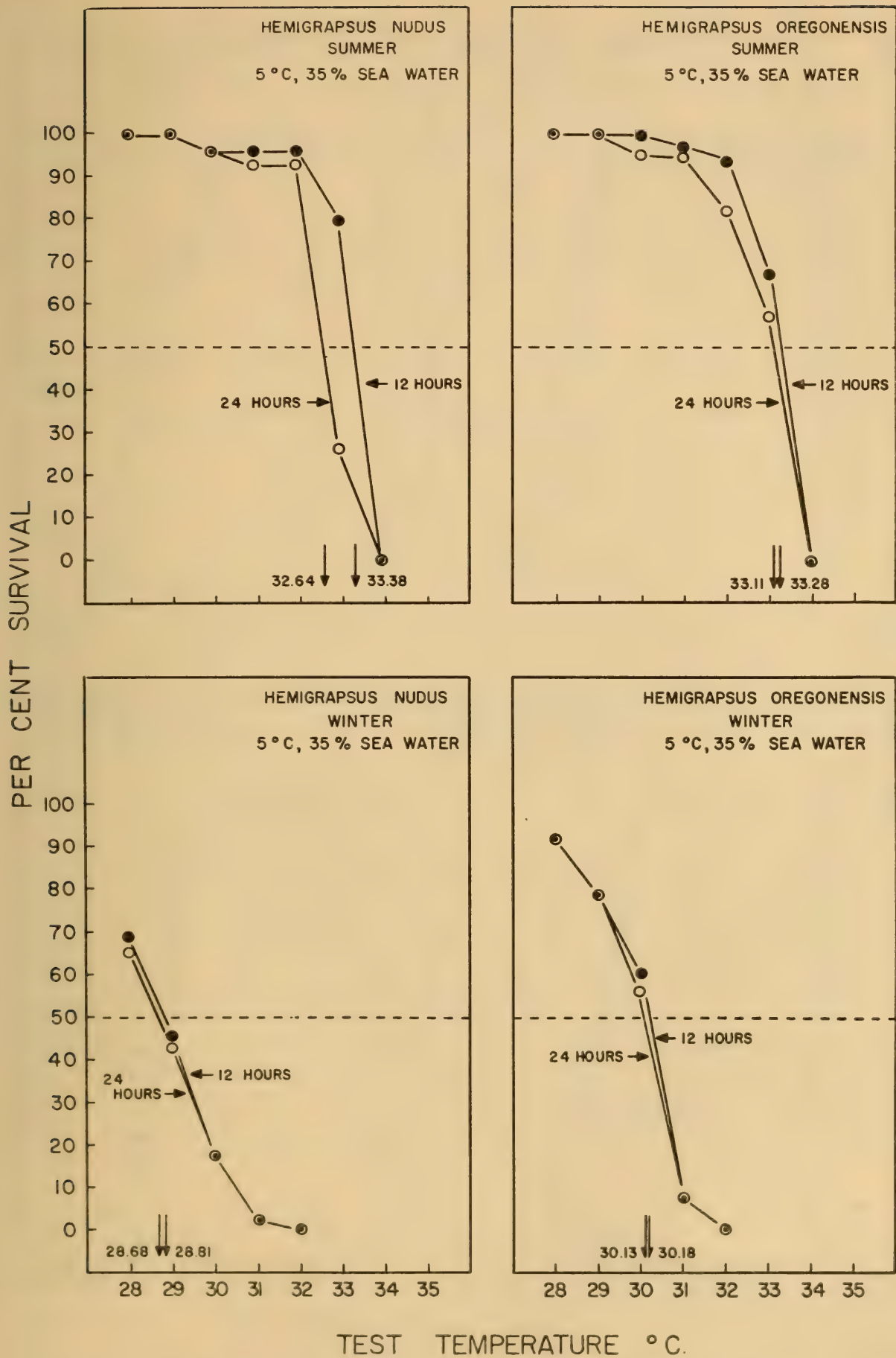


FIGURE 3. The influence of a previous history of 5° C., 35 per cent sea water on the 50 per cent survival values at 12 (●) and 24 (○) hours. Both species in summer and winter are shown. Each point represents the average survival of at least thirty animals.

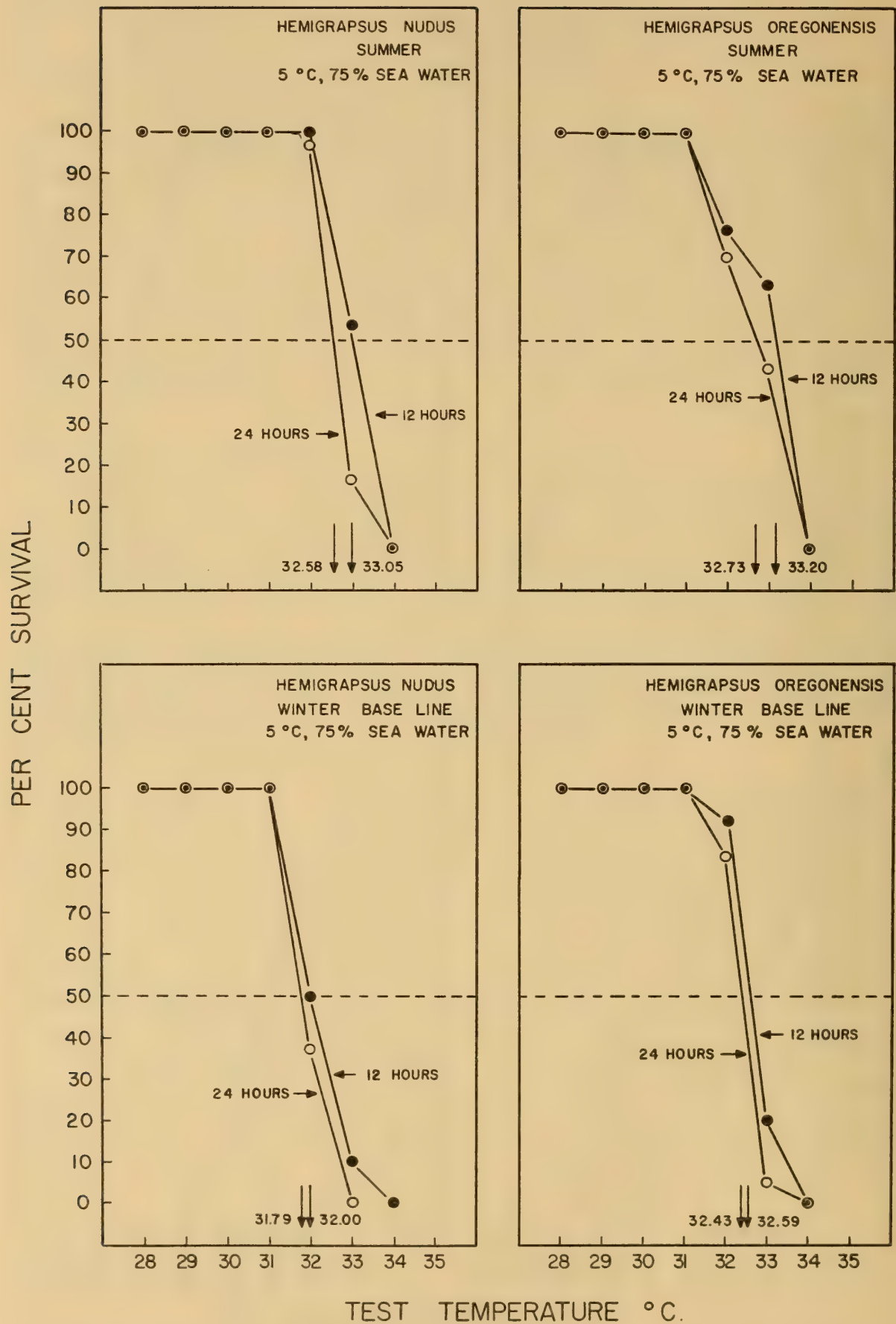


FIGURE 4. The influence of a previous history of 5° C., 75 per cent sea water on the 50 per cent survival values at 12 (●) and 24 (○) hours. Both species in summer and winter are shown. Each point represents the average survival of at least thirty animals.

TABLE I

The change in 50 per cent survival temperature with various temperature-salinity combinations

		SUMMER		WINTER	
		<i>H. nudus</i>	<i>H. oregonensis</i>	<i>H. nudus</i>	<i>H. oregonensis</i>
20° C., 35% sea water	12 hr.	32.95° C.*	33.43*	32.54	33.38
	24	32.50*	33.16*	32.22	33.04
20° C., 75% sea water	12 hr.	33.62	34.50	34.32	34.40
	24	33.45	34.38	33.45	33.66
5° C., 35% sea water	12 hr.	33.38	33.28	28.81	30.18
	24	32.64	33.11	28.68	30.13
5° C., 75% sea water	12 hr.	33.05	33.20	32.00*	32.59*
	24	32.58	32.73	31.79*	32.43*

* Base line tolerances in the two seasons, summer and winter.

of variance on summer data indicated that *H. oregonensis* was the more resistant at lethal temperatures, $P = .01$. The lower resistance is surprising in *H. nudus* as the species is more commonly found at a higher tide level, and it would be expected, *a priori*, that a greater tolerance for high temperatures would be of more advantage to that species. Also, the similarity of the 50 per cent survival temperatures for 12 and 24 hours within each species was consistent throughout. At each test temperature the values were very close, and in many cases were identical. Table I summarizes the results.

Salinity had a pronounced effect on the amount of variability that was obtained in a series of replications of one of the experimental conditions. Whether the acclimation temperature was 20° C. or 5° C., but particularly with the low acclimation temperature, the low salinity (35 per cent sea water) resulted in much more variable survival percentages at the test tolerance temperatures than those found with the higher salinity. Also, the temperature at which 100 per cent survival occurred was a much more tenuous point with the lower salinity. This can be seen readily in any of the low salinity graphs, as there is a gradual slope to the final breaking-off point, whereas with the 75 per cent salinity, the break is abrupt, directly from 100 per cent. It should be noted that the difference in temperatures between the points where 100 per cent or close to 100 per cent survival is found for 24 hours and complete mortality is very slight.

Effects of size, sex, crowding, and moulting

All weights of crabs did not appear to have the same resistance to the high test tolerance temperatures. Smaller animals, in both species, seemed slightly more tolerant than larger ones. There was no direct relationship between weight and time to death, but results indicated that if any animal lived, it was usually less than 1 gram. This seemingly greater tolerance in the smaller crabs was

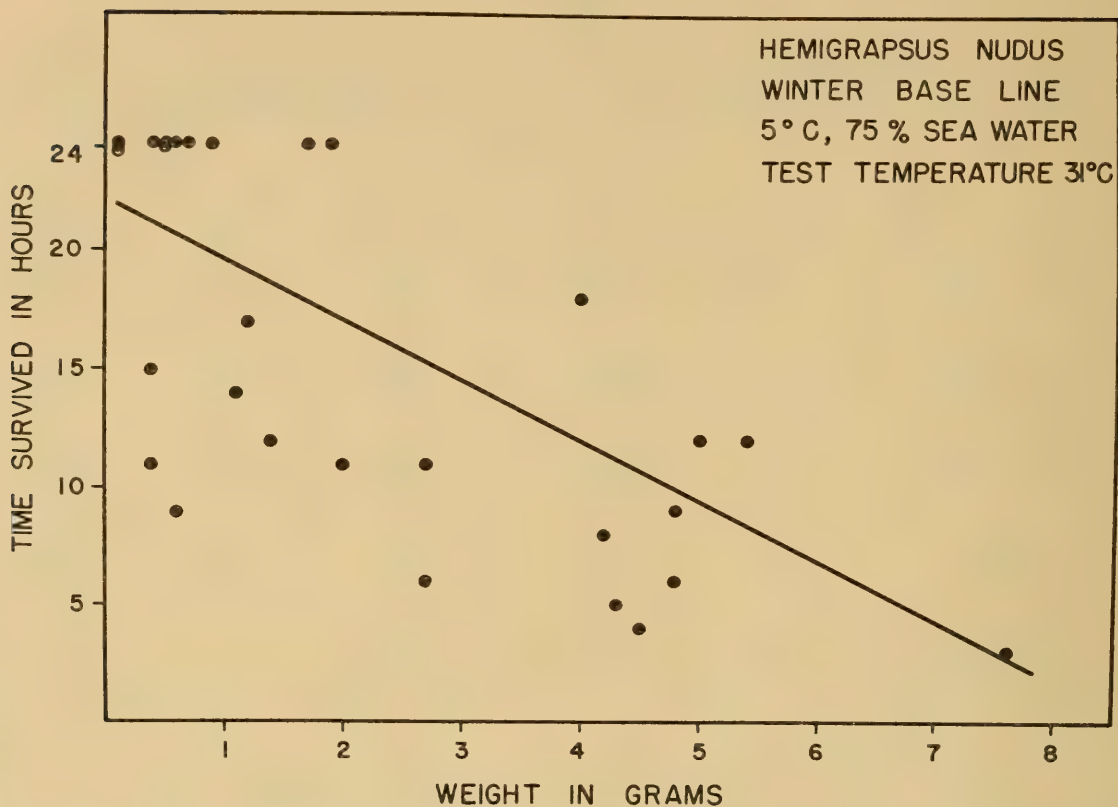


FIGURE 5. The effect of weight on the time survived in hours at 31° C. in *Hemigrapsus nudus*. The animals had a previous history of 5° C., 75 per cent sea water. Each point represents one animal. The regression line is eye-fitted.

prevalent when crabs from all temperature-salinity combinations were tested. One graph was chosen to demonstrate this phenomenon (Fig. 5).

As mentioned previously, both males and females were used in the experiments when the sex was found to have no influence on the resistance to the high temperatures. To the contrary, crowding, that is more than 5 animals per 4 liters of water, caused a marked lowering of resistance to high temperatures. A minimum value of 0.333 liters of sea water per 1-gram live animal weight was found to be satisfactory. When less water was allowed, by including more animals per dish, dying animals apparently affected the water even though they were removed immediately upon death, because those remaining were more apt to die (Fig. 6). Thus, with 5 animals per dish, the 50 per cent survival temperature for 12 hours was 32.95° C., with 10–15 animals per dish, 30.68° C., and with 19–28 animals, 27.86° C. This crowding effect existed only at the high test tolerance temperatures. As was mentioned in the previous section, when there were at least 35 animals per dish at the acclimation temperature of 20° C., no mortality due to crowding was noted.

Since only crabs with a fully hardened carapace were used in the experiments, the effects of moulting on the temperature tolerance were determined from those animals which moulted during the 24-hour course of the experiment. These animals invariably died, even at a temperature where 100 per cent survival normally occurred. A possible change in permeability of the carapace after moulting may have rendered the crabs less resistant to high temperatures.

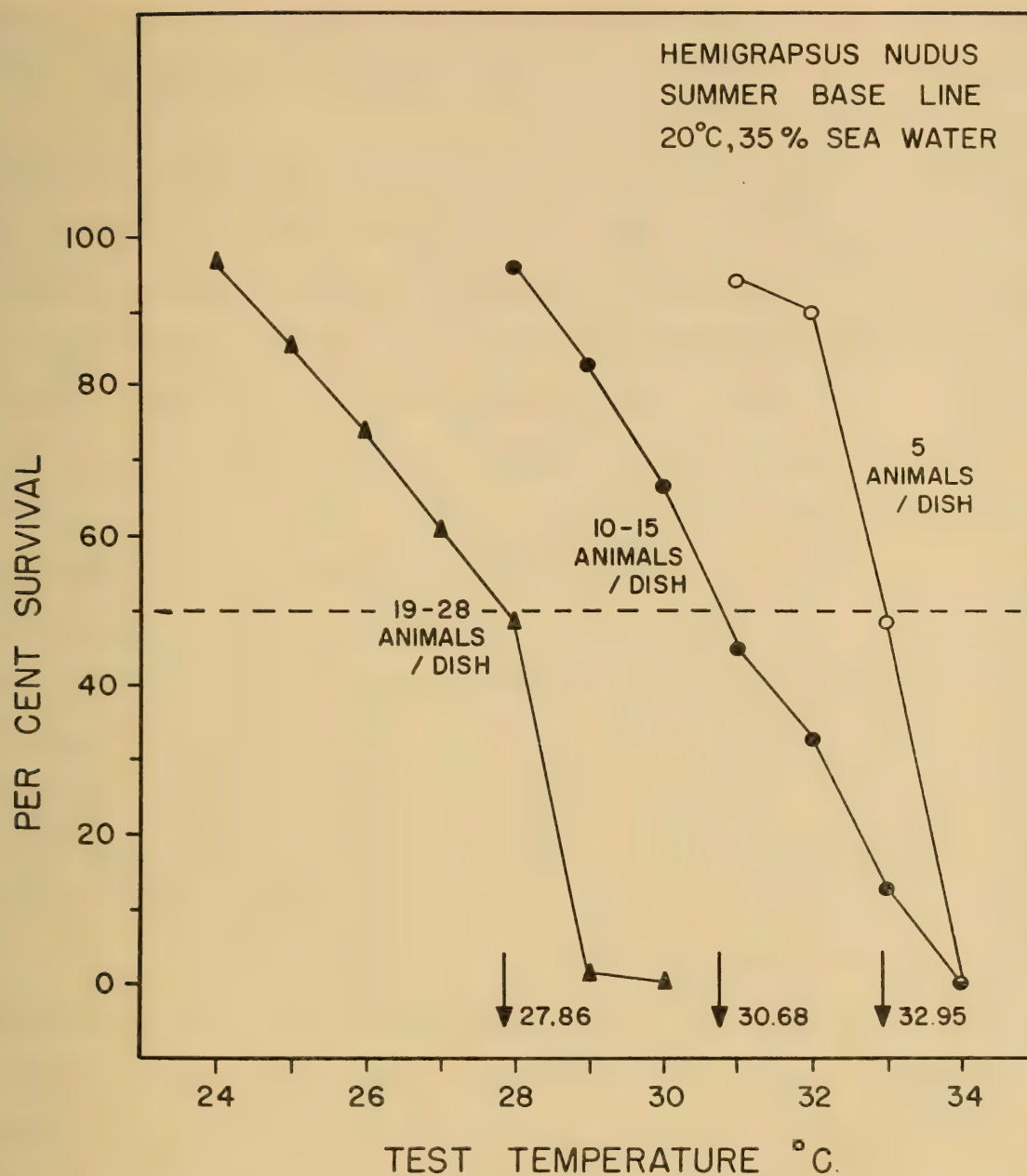


FIGURE 6. The influence in *Hemigrapsus nudus* of differing densities of crabs per 4 liters of sea water on the 50 per cent survival temperature at 12 hours with a previous history of 20° C., 35 per cent sea water: 5 animals per dish (○), 10 to 15 animals per dish (●), 19 to 28 animals per dish (▲). Each point represents the average survival of at least thirty animals.

DISCUSSION

Acclimation to a high temperature, with resulting increase in the high temperature tolerance of the species, has been demonstrated many times (Sumner and Doudoroff, 1938; Brett, 1946; Mellanby, 1954; Spoor, 1955; McLeese, 1956). The two species of crabs, *H. nudus* and *H. oregonensis*, studied here, would seem to be no exception. Temperature tolerance in conjunction with salinity has been studied less extensively, and it was of interest to find that the salinity had a very marked effect on the temperature tolerance of the species. Although results from

the survival curves occasionally differed in summer animals as opposed to winter animals, the trend with regard to tolerance in any particular temperature-salinity combination was the same in both seasons and both species, but there was a definite species difference.

During the summer months, the environmental temperature is high and the salinity is low. When both species of winter animals were acclimated to these conditions, it was found that these acclimated animals had a tolerance at high temperatures which was approximately as great as that found in the summer animals. Likewise, these winter animals when acclimated to the high salinity as well as to the high temperature, demonstrated in both species, again a temperature tolerance very similar to that found in summer animals acclimated to these same conditions, by far the most favourable combination to resist heat death. It is evident that these animals can regain their tolerance to high temperatures very rapidly after a low temperature history by acclimation in the upper part of the physiological temperature range. This rapid gain of heat tolerance has been documented many times. Mellanby (1954) reported that the heat coma point is shifted with experimental acclimation at the upper end of the tolerable range in the mealworm, *Tenebrio molitor* and mosquito, *Aedes aegypti*. The gain in tolerance was rapid, as twenty hours' acclimation produced as much change as longer acclimation periods. Spoor (1955) found complete gain of heat tolerance in the crayfish in less than 24 hours with an approximate 18° C. change in temperature (4° C. to 23° C.). Ohsawa (1956a) determined that gain in heat tolerance by the periwinkle was complete in less than 48 hours when acclimated to 30° C. from 10° C. One exception to a fairly rapid gain in heat tolerance is a recorded time of about twenty-two days for total acclimation in the American lobster when transferred from 14.5° C. to 23° C. (McLeese, 1956). McLeese suggested that the reason for this unusual length of time is that the lobster has a long latent period before onset of demonstrable acclimation, in this case ten days.

A rapid gain of heat tolerance would be of extreme advantage to most intertidal animals. In those animals permanently submerged, a quick augmentation would appear to be of little direct advantage, as bodies of water only slowly change in temperature. For intertidal animals, the increase in temperature may be as much as 10° C. over a period of a few hours in the tidal rhythm. At Spanish Bank, the collecting area for *H. nudus* and *H. oregonensis*, tide pools have been recorded at 26° C. to 28.5° C. In this case, a rapid gain in tolerance, particularly over a period of a few hours, would be most advantageous. Probable increase in salinity, due to evaporation in tide pools during the low tide period in summer months, aids the animal in resisting the high temperatures, as experimental results have indicated that high salinity provides the most favourable environment.

Regarding the low temperature series, the results can not be interpreted as readily. The seasonal difference is marked when compared with the difference found in the high temperature series. The change was consistently to a lower temperature for the 50 per cent survival value in the winter animals. For the winter base line, 5° C., 75 per cent sea water, a drop of about 1° C., from summer animals acclimated to these same conditions, was found in both species at 12 and 24 hours. With the low salinity, low temperature combination, loss in heat tolerance is much greater. In this low temperature series it is clear that the seasonal

alteration in the field environment produces a noticeable reduction of tolerance in the winter animals to high temperatures.

Seasonal change in heat tolerance of a species has been noted by Edwards and Irving (1943) who found a 10° C. difference in the death point of *Emerita talpoida* with summer animals higher than winter ones. Brett (1944) found a change in upper lethal temperatures in Algonquin Park fishes from spring to fall. In most cases, the species studied have been acclimated either solely to various temperatures in the laboratory and the temperature tolerance studied, or the tolerance has been tested at different seasons with no laboratory acclimation. Examples of seasonal comparisons in thermal resistance with the same acclimation temperature in summer and winter are tolerance studies on several species of fresh water fish (Hart, 1952), planaria (Schlieper and Bläsing, 1953, as reported in Fry, 1958) and the rainbow trout (Keiz, 1953). These species were more tolerant of high temperatures in the summer months. Ohsawa (1956a) found that summer periwinkles responded at a higher temperature than winter animals when tested over the same range. Prior to the test, the summer animals had a 48-hour history at 10° C. which corresponded to winter field conditions.

As in all acclimation experiments in this investigation, the summer animals of the low temperature series were kept one week before survival was determined at the test tolerance temperatures. There was no change in the tolerance over the testing interval, seven to thirteen days. This period of approximately two weeks was in agreement with that found necessary by other investigators to demonstrate laboratory acclimation to low temperature. It was evident from experiments on animals collected in winter months that summer animals acclimated to winter field conditions of temperature and salinity in no way approached the reduced tolerance found in winter animals. Likewise, summer animals acclimated to low temperature plus low salinity had a much greater tolerance than was found with the corresponding acclimation condition in winter animals. Insufficient length of time for acclimation is an obvious possibility, but it would be thought that some change would have shown in the tolerance over the period of two weeks. A long latent period before the onset of acclimation to the low temperature is feasible, as a latent period of 10 days was reported in acclimation to a higher temperature in the lobster (McLeese, 1956). Another possibility is that some other factor or a combination of factors influences the acclimation to low temperatures but not to the reverse, as winter animals which were acclimated to summer conditions gave approximately the same results as summer animals. Dehnel (1958) has shown in these two species of crabs, *H. nudus* and *H. oregonensis*, that a simulated seasonal variation of light in the laboratory has a pronounced effect on metabolism measured by oxygen consumption. It is feasible that summer animals would never have the same temperature tolerance as that found in the winter animals solely by acclimating them to winter temperature and salinity conditions. Hoar (1956, p. 365) from experiments on goldfish "... concluded that the seasonal variations in thermal tolerance previously noted in fish maintained under constant temperature conditions are photoperiodically controlled. . . ." It is hoped that future experiments will determine this relationship more clearly in these crabs.

Recently, further experiments were carried out on this problem. There was

an attempt to acclimate summer crabs of both species to the low salinity, low temperature combination to determine whether results similar to those found in winter could be obtained. This combination was chosen as acclimation to this temperature-salinity condition resulted in greatly reduced tolerances in winter. The summer animals were acclimated for a total of thirty-one days in some experiments. Resistance appeared to be slightly less when compared with tolerances found after shorter periods of acclimation, but was not as low as those found in winter. Also, experiments were done where the animals had an eight-hour photoperiod corresponding to light conditions in winter with the above temperature-salinity relation. Results again indicated a slightly reduced tolerance but did not appear to differ from those of animals kept in complete darkness. Since two weeks' acclimation in the summer animals did not bring about a change over the testing period, it is important to note that animals cannot be said to acclimate or not acclimate to a parameter merely because there is no evident change.

In nearly all instances low salinity resulted in a lower value for the 50 per cent survival temperature than with high salinity, whether the temperature was 5° C. or 20° C. Gross (1957) gives values for *H. nudus* and *H. oregonensis*, calculated from Jones (1941), which indicate the osmotic gradient maintained by these two species when placed in various concentrations of sea water. The crabs are isotonic to the external environment at 100 per cent sea water (based on 34.6‰). At 75 per cent sea water there is an extremely slight gradient maintained, about 2.5 per cent sea water in *H. oregonensis* and 8.0 per cent in *H. nudus*. The gradient maintained at 35 per cent sea water, however, is about 36 per cent in both species. These values cannot be applied directly to the present experiments, but certainly they can indicate the trend. The maintenance of this gradient presumably results in more work being done by the animal at the lower salinity, and in a greater stress placed on it than at 75 per cent sea water where the gradient is only a few per cent. Additional evidence which suggests more osmotic work is performed at the lower salinity in these crabs is a 22 per cent increase in respiratory rate at 35 per cent over that found at 75 per cent sea water in a 1.0-gram animal measured at 10° C. (Dehnel, 1960). It is probable that the lower 50 per cent survival temperatures with the lower salinity is due to the increased metabolic work necessary at that salinity. The lethal or near lethal temperatures in the test tolerance experiments presumably alone are causing a marked strain on the metabolic activity of the crabs and the additional strain of maintaining a large gradient between the blood and the external medium in the low salinity results in the animal dying at a lower temperature.

In the present investigation, low salinity, combined with low temperature, particularly in the winter animals, was the most disadvantageous acclimation combination for withstanding the test tolerance temperatures. Wikgren (1953) showed the greater loss of ions with temperature decrease when transferred from tap to distilled water. He thought that this greater loss at the lower temperatures could be assigned to depressed absorption of ions and chloride.

A similar relation between salinity and temperature was found by Broekema (1941) in the shrimp, *Crangon crangon*. The salinity optimum for length of life depended on the temperature. With a rise in temperature, the salinity optimum was reached by a downward shift in the salinity. High temperatures generally

were less favourable for survival than low, but low salinity was endured better with a higher temperature. This was similar to the pattern in the two species of crabs studied in these experiments; with low salinity, temperature tolerance was increased when there was a previous high temperature history rather than a low, particularly in winter animals. In addition Broekema showed that the difference between the internal blood concentration and external concentrations in the shrimp was greater at high than at low temperatures and suggested that at low temperatures the limits of life are exceeded sooner than when the temperature is high. Jones (1941) demonstrated a higher osmotic pressure in the blood of *H. nudus* and *H. oregonensis* with a higher temperature, signifying an increase in the degree of regulation. Thus, with a difference in temperature acclimation at the low salinity it is probable that the difference in sustained gradients could carry through to the temperature tolerance tests, and thereby affect the lethal point. Jones, however, showed a great variation in the osmotic pressure of the blood when these crabs were near death from air exposure and felt that this tended to refute a certain lethal osmotic pressure as the primary cause of death.

Broekema's findings, demonstrating that shrimp in a high salinity lived longer at a low temperature, at first appear contrary to the results of this investigation, where the most favourable history for resisting high temperatures was one of both high temperature and high salinity. It must be remembered, however, that the criterion for optimal conditions was quite different, ability to resist lethal temperatures in this investigation, whereas in Broekema's experiments the length of life was the factor. The results cannot be compared directly, but it is felt that they both show the same trend, particularly with respect to low salinities.

Kinne (1958) reported the shifting of heat tolerance in three species of animals, the polychaete *Nereis diversicolor*, the amphipod *Gammarus duebeni*, and the isopod *Sphaeroma hookeri*. The salinity of the pond from which all three species were collected was about 12‰, and in all three species when kept at salinities below this, heat resistance is lowered. Increased heat resistance results in animals from the higher salinities, above 12‰. Kinne suggested that the alteration of water and ion balance resulting in increased water content at extremely low salinities decreases the heat tolerance; the lowered water content at higher salinities favourably affects the resistance to high temperatures. The range of test salinities was beyond the limits of regulation in *Gammarus duebeni* from values given by Verwey (1957) and at the upper salinities in *Nereis diversicolor* (Smith, 1955b). Another explanation than alteration of water content may be necessary when similar changes in tolerance occur over the physiological regulatory range where variation is slight. Since the salinity of the pond where the animals were collected was only 12‰, this would result in a fairly large sustained gradient at least in two of the three species for which data are available. The salinities could cause increased susceptibility to lethal temperatures. The higher salinities presumably within limits would approximate more nearly natural conditions in the body fluids of the animals, to provide a more favourable environment for survival. Another species studied by Kinne (1956), the hydroid *Cordylophora caspia*, has slight osmoregulatory abilities, and again was found to withstand a high temperature better at a high salinity than at lower ones. Possibly the explanation here could be that the higher salinity is nearer the concentration of body fluids of the hydroid,

resulting in a more favourable environment to resist a stress, in this case, high temperature.

The geographic limitation of the polychaete *Nereis* is related to the salinity-temperature balance of its environment. The range of *Nereis diversicolor* in the Baltic Sea apparently is limited by the colder parts of the year when it could not withstand the low salinity expressed as a chlorinity of 4 grams per liter in which it is able to regulate adequately in the warmer summer months (Smith, 1955a). Studies on *Neanthes lighti* indicated that it was able to survive in fresh water in Lake Merced, California, because of its viviparous mode of reproduction where the young are sufficiently developed at birth that they are able to osmoregulate, and that the species is not exposed to severe winter cold. Laboratory experiments showed that the ability to regulate in water of a chlorinity of 1.0 to 0.09 grams per liter was inhibited at 1.5° C. but possible at 13° C. in worms tested in vitro (Smith, 1957).

Animals acclimated to temperatures in the upper part of their physiological temperature range show a remarkable similarity in lethal temperatures. A few comparisons for estimated 12 hours median tolerance among animals acclimated to approximately 20° C. and a favourable salinity, if marine forms, are given in Table II. For the most part the lethal temperature is at least 30° C. In the species difference found in this investigation, the greater thermal resistance of *H. oregonensis* is surprising since it occupies a lower level on the beach. Within the same species, *Littorina litorea*, the individuals collected at high tide level had greater resistance to lethal temperatures than those collected a few yards away at low tide level (Gowanloch and Hayes, 1926). Ohsawa (1956b) determined species difference for the periwinkles *Nodilittorina granularis* and *N. vilis*. The latter occurs in the upper zone of the wide range occupied by *N. granularis*, which was found to be the more tolerant at lethal temperatures, a similar result to that found with *H. nudus* and *H. oregonensis*.

In most animals, then, and possibly all, a previous high temperature history increases heat tolerance and a low one decreases it. Along with this is the influence of salinity where low salinities almost invariably are endured better at a higher temperature, and high temperature, high salinity combined being most favourable for withstanding upper lethal temperatures. Low salinity, low temperature generally is least favourable for length of life and for heat tolerance (Fig. 7).

TABLE II

A comparison of tolerances to high temperature in several species

Species	12 hour median tolerance, ° C.	Source
<i>Hemigrapsus nudus</i>	33.62	—
<i>H. oregonensis</i>	34.50	—
<i>Orconectes rusticus</i>	36.4	Spoor, 1955
<i>O. propinquus</i>	35.0	Bovbjerg, 1952
<i>Cambarus fodiens</i>	35.0	Bovbjerg, 1952
<i>Homarus americanus</i>	28.4–30.5	McLeese, 1956
<i>Hyalella azteca</i>	33.0	Bovee, 1949
<i>Ameiurus nebulosus</i>	33.4	Brett, 1944
<i>Carassius auratus</i>	34.5	Fry, Brett and Clawson, 1942

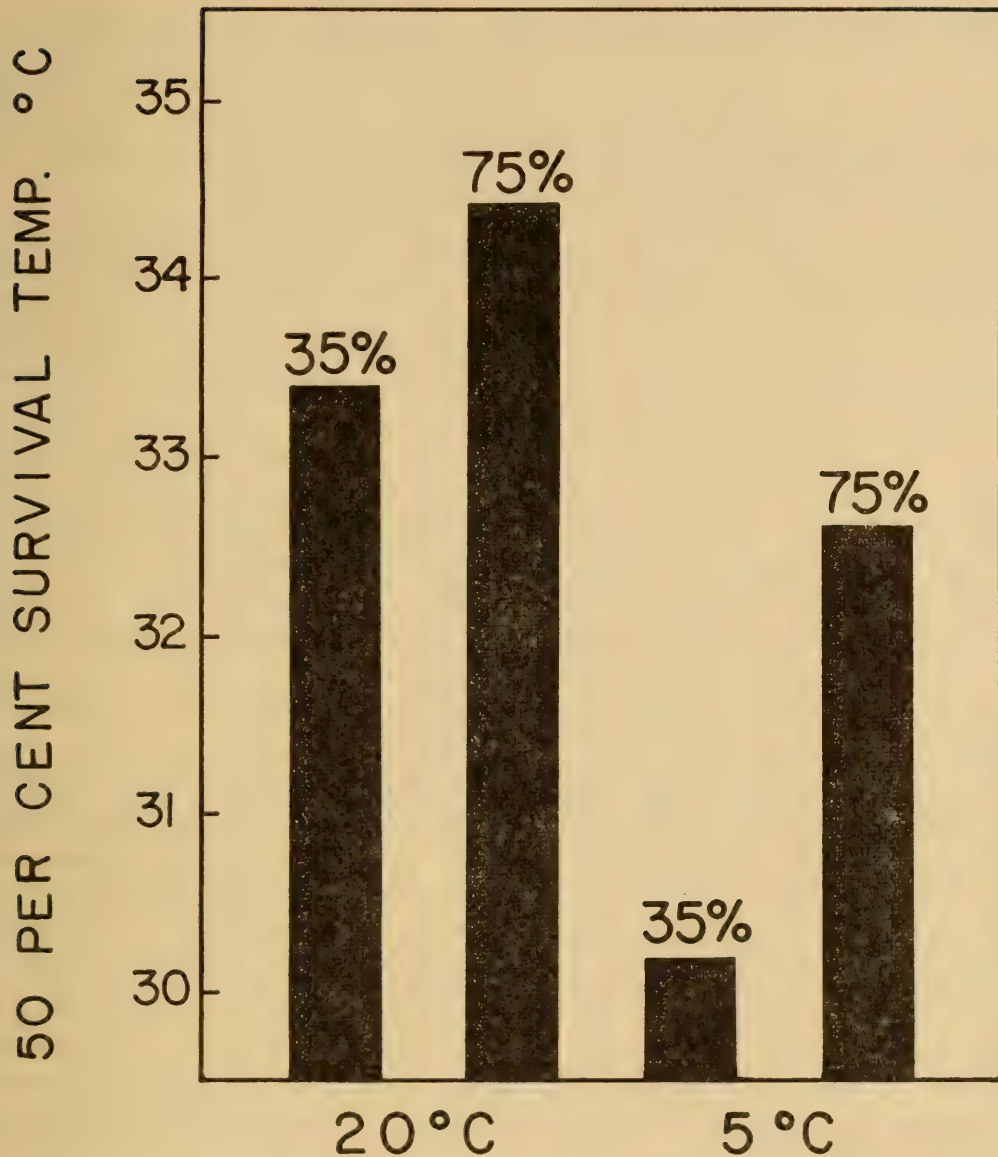


FIGURE 7. Median tolerance temperatures for *Hemigrapsus oregonensis* for 12 hours with previous histories of 20° C. or 5° C. and 35 per cent or 75 per cent sea water. Animals were collected in winter.

The effect of size and sex on heat tolerance was found to vary in different animals reported in the literature. In *H. nudus* and *H. oregonensis*, smaller animals seemed to be slightly more resistant to the high test tolerance temperatures than larger individuals. There was no difference in tolerance between the sexes. Edwards and Irving (1943) found no difference in tolerance in *Emerita talpoida* between males and females, but larger animals seemed slightly more resistant than small ones. In lethal temperature experiments on young speckled trout, *Salvelinus fontinalis*, size did not affect time to death at high temperatures in the same age group (Fry, Hart and Walker, 1946). Hoar (1955) found an increased tolerance at 1°–2° C. in goldfish with an increase in size from 3 to 7.5 cm. In the crayfish, sex and size did not affect heat tolerance (Spoor, 1955). McLeese (1956) concluded that in the size range of lobsters studied, from 21 to 28 cm.,

there is identical response to upper lethal temperatures. Kinne (1958) showed a decreased tolerance in female *Gammarus duebeni* to high temperatures, and increased tolerance in smaller (younger) animals of both sexes. In *Sphaeroma hookeri*, smaller males appeared more resistant than larger ones. There was apparently no sex difference in this species. Thus, it is seen that no conclusions can be drawn regarding the effect of size or sex on heat resistance.

The amount of water available to each animal during the experiments testing heat tolerance was found to influence markedly that tolerance even though animals were removed at death. At least 0.333 liter of water per gm. live animal weight per 12 hours was necessary to prevent death from crowding. The amount of water provided per gm. varies widely in different investigations. Gowanloch and Hayes (1926) tested 10 to 15 periwinkles per 250 cc. of water (0.250 liter), less water than that found necessary in the lethal temperature experiments on these crabs. Brett (1952) tested 40 fish (average weight about 1 gm.) in a lethal bath twenty-two inches square by eleven inches deep. This gave about 2.2 liters per gm. Also the exchange of water was equal to the volume of the tank every 24 hours. Spoor (1955) provided at least 0.1 liter of water for each animal being tested. The size range was from 17 to 42 mm. McLeese (1956) tested 10 lobsters at each constant temperature. Taking a value of 400 gm. as an average weight of the experimental animals, only about 14 cc. (0.0139 liter) at the most was allowed per gram. This was very much less than would be expected if the amount for the decapod Crustacea found in this investigation can be related. A small flow of water into the tank presumably allowed for some exchange of water, but no values are given. Also, the lethal temperatures are low both in comparison with other Crustacea and particularly with the values obtained by Huntsman (1924) for stages IV and V in the lobster. An estimated lethal temperature of about 34° C. is greater than that obtained by McLeese under the most favourable acclimation conditions. Too many animals per volume of water may account in part for results. In many cases in the literature, the volume of water used in the experiment is not indicated, but presumably tests were conducted to ensure a satisfactory density of animals. It is probable that the volume needed varies in different species.

Moulting during the 24 hours of the experiment was found to have an adverse effect on high temperature tolerance in *H. nudus* and *H. oregonensis*. Crayfish moulted successfully in temperatures from 12° C. to 36° C.; the stage in the moult cycle had no effect upon heat tolerance (Spoor, 1955). To the contrary, McLeese (1956) showed that the average survival time for soft-shell lobsters was less than that for hard-shelled individuals. As results from various workers differ, nothing definite can be concluded about a relationship between moulting and death at high lethal temperatures.

SUMMARY

1. The influence on heat tolerance was determined of seasonal change and laboratory acclimation to various temperature-salinity combinations, for two species of grapsoid crabs, *Hemigrapsus nudus* and *H. oregonensis*.

2. There was a seasonal change in 50 per cent survival in both species when base lines from summer and winter were compared.

3. A definite species difference in tolerance to high temperatures was found to exist, but both species reacted similarly to any particular temperature-salinity combination.

4. Acclimation to a high temperature generally increased the resistance to lethal temperatures whereas acclimation to low salinity generally decreased it. High temperature, high salinity was the most favourable combination to withstand the high test tolerance temperatures.

5. Gain in heat tolerance whether the salinity was low or high was rapid, less than one week.

6. Winter tolerances with both low and high salinities in the low temperature series were not demonstrated in the laboratory with summer animals acclimated to these same conditions. Various reasons are suggested which might explain this apparent discrepancy.

7. Moulting during the test tolerance experiments adversely affected the resistance. The number of animals per dish at each test temperature had a pronounced effect on tolerance. The sex of the crabs did not affect the survival, but smaller animals appeared to be slightly more resistant.

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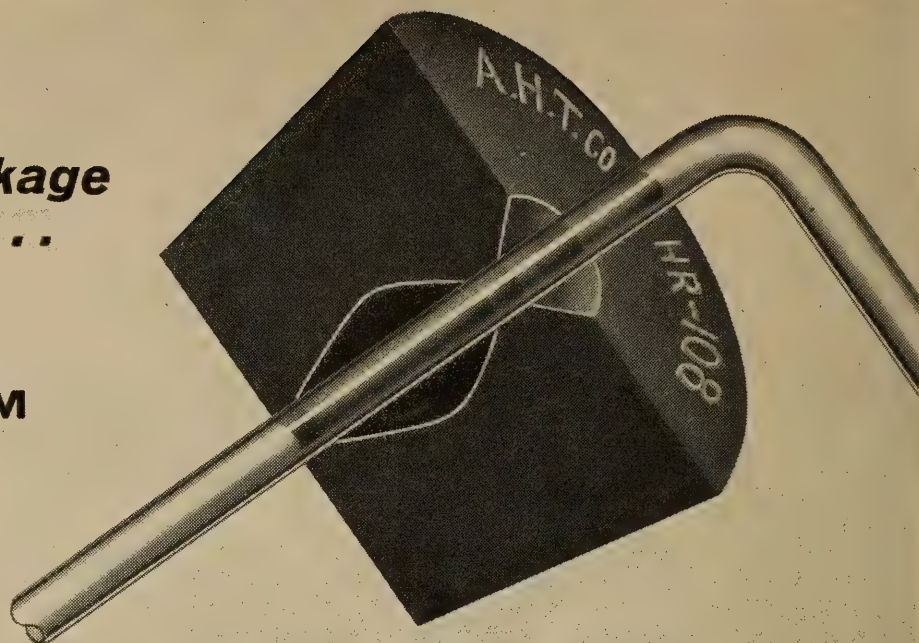
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THE BIOLOGICAL BULLETIN

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METABOLISM OF SULFUR AMINO ACIDS IN MYTILUS EDULIS AND RANGIA CUNEATA¹

KENNETH ALLEN² AND J. AWAPARA

Biology Department, Rice Institute, Houston, Texas

Sulfur amino acids are important metabolites in living systems. Their metabolism is well known in mammals—the reactions involved in their breakdown are known and so are the end products formed. In invertebrates, however, less is known about the metabolism of sulfur amino acids. It is known that some marine invertebrates contain in their tissues large amounts of taurine, (Kelley, 1904; Henze, 1905; Mendel, 1904; Kossel and Edlbacher, 1915; Okuda, 1920; Ackermann *et al.*, 1924) but not much is known of its origin or its role in the animal. Recently, several papers have been published in which the probable origin of taurine is mentioned. In one paper, Shibuya and Shunji (1957) reported that hypotaurine, a precursor of taurine, was present in *Septifer virgatus*; Ouchi (1959) claimed that hypotaurine was present in numerous species of molluscs. Hypotaurine, first found in the rat by Awapara (1953), is formed from cysteine sulfinic acid and converted to taurine as demonstrated by Awapara and Wingo (1953). Taurine, then, could probably be formed in invertebrates by the same sequence of reactions as in the rat. Cotty *et al.* (1958) studied the metabolism of sulfur amino acids in *Musca domestica* and concluded that methionine serves as a precursor of taurine.

In a recent paper, we reported that taurine is present in all marine invertebrates studied, but was absent or not detectable in fresh water and terrestrial molluscs (Simpson, Allen and Awapara, 1959). This comparative study did not tell us whether sulfur amino acids are metabolized differently by marine molluscs and fresh-water molluscs. The end products of sulfur metabolism could be different; we needed to know more about the intermediate steps. We have performed a number of experiments on *Mytilus edulis*, which is known to have large amounts of taurine, and on *Rangia cuneata* which has none detectable by paper chromatography. The results of these experiments show that both can convert methionine to cysteine, probably in the same manner as mammals do, and that cysteine can be oxidized to various products some of which can be decarboxylated to give taurine.

¹ This work was supported by grants from the Robert A. Welch Foundation, Houston, and from the National Institutes of Health, U. S. Public Health Service.

² The data reported here is part of the Ph.D. thesis of Kenneth Allen.

MATERIALS AND METHODS

Animals

M. edulis was obtained from the Marine Biological Laboratory, Woods Hole. The animals were put in sea water cooled to 5° C. and kept there until they were used. *R. cuneata* was collected from the San Jacinto River, Harris County, Texas. They were kept in large aerated aquaria.

Chemicals

S³⁵-methionine, S³⁵-cystine and S³⁵-sodium sulfite were obtained from Abbott Laboratories, Oak Ridge. They were chromatographically pure. S³⁵-taurine was prepared from S³⁵-sulfite and β -bromoethylamine hydrobromide according to the method of Cortese (1943). The crude taurine was purified by chromatography on a column of Dowex 50 in the H⁺ form. The final product was chromatographically pure. Cystine was reduced to cysteine according to the method of Lucas and Beveridge (1940).

Administration of compounds

Solutions of S³⁵-methionine, S³⁵-cysteine and S³⁵-taurine were prepared and administered to both *M. edulis* and *R. cuneata* in the following manner: the shell of *M. edulis* was opened and kept open with wound spreaders. The foot was located and 1 ml. of the solution carefully injected into the foot; a 1-ml. syringe with a 22-gauge needle was used. This method was suggested by Dr. T. W. Potts, of the University of Birmingham, England. *R. cuneata* was injected through a hole cut carefully in the shell; the needle was inserted through the mantle and gill into the fleshy part of the viscera. After the injection the animals were returned to aquaria containing water of the appropriate salinity and temperature.

Extraction and fractionation of amino acids

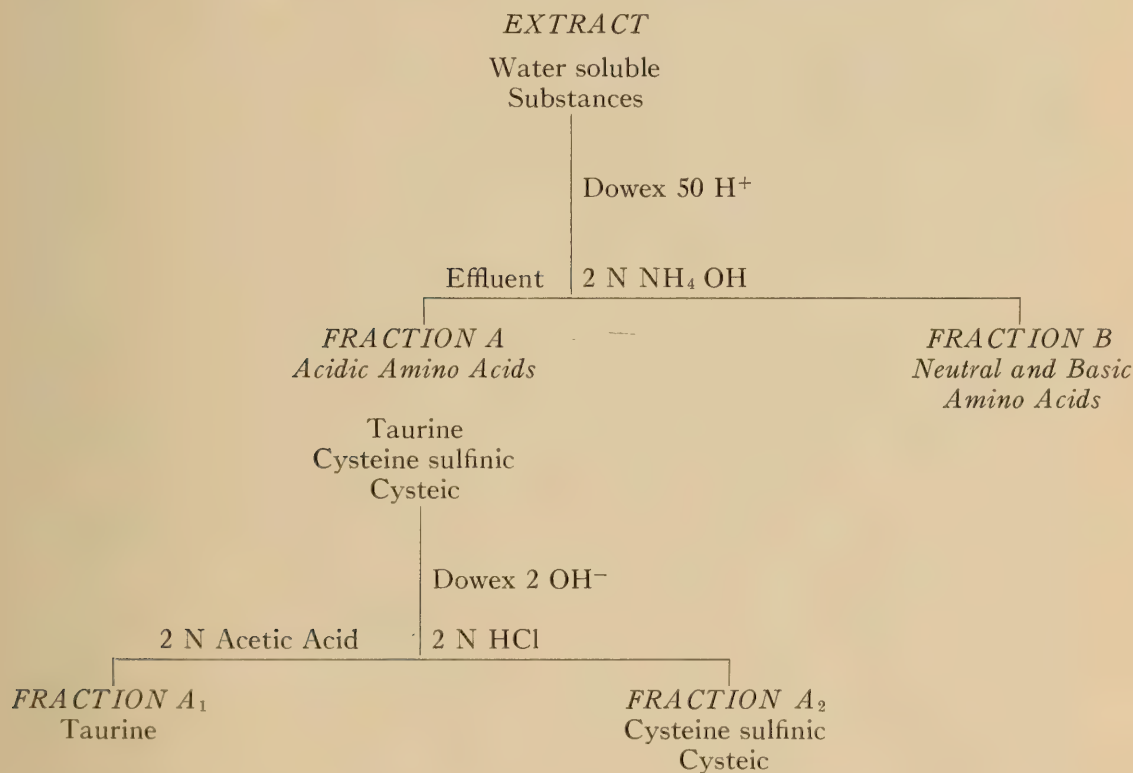
Extractions were carried out by the method of Awapara (1948). The extracts were used in some instances for paper chromatography and in others for isolation of intermediates. Most of the time the extracts were fractionated into various groups, using ion exchange resins according to the scheme shown in Table I. According to this scheme the extracts containing one gram of tissue per cc. were first put through a column (10 cm. $\times$ 1 cm.) of Dowex 50 in the H⁺ form (200–400 mesh). The column was washed with distilled water until the effluent became neutral. All the anions and non-polar compounds were washed through by this procedure, along with taurine, cysteic acid and cysteine sulfinic acid. All other amino acids were eluted from the resin bed with 25 ml. of 2 N NH₄OH; the acid effluent (A) and the NH₄OH eluate (B) were evaporated to dryness; the residues redissolved in a small amount of water. Fraction A, which contained taurine, cysteic acid, cysteine sulfinic acid and sulfate, was fractionated using a strong anion exchange resin—Dowex 2 in the OH⁻ form. The solution was put through a small column of the resin (5 cm. $\times$ 1 cm.) and washed with 50 ml. of boiled and cooled distilled water. Elution was carried out in two stages: (1) with 10 ml. of 2 N acetic acid to remove taurine, and (2) with 10 ml. of 2 N HCl to remove

cysteic and cysteine sulfinic acids. Fractions A_1 and A_2 were evaporated to dryness; the residues were dissolved in small amounts of water and the evaporation repeated to drive off most of the acid. Finally the residues were taken up in small portions of water and stored for further work.

Identification of products

Intermediates and final products were identified by several methods: (a) The various fractions were chromatographed on paper; radioautographs were made of these papers using x-ray film. After a three- to five-week exposure the papers

TABLE I
Fractionation of extracts by means of ion exchange resins



were developed with ninhydrin. The ninhydrin spots were matched against the blackened areas on the film. Tentative identifications were thus made on the basis of R_f values. (b) Some of the fractions were chromatographed on paper strips and the strips analyzed for radioactivity in a continuous strip counter. The position of the peaks was used as criterion for identification. Also, chromatography on strips was carried out using the various unknown fractions but mixed with known substances suspected to be present in the fractions. A perfect overlap between the peaks and the ninhydrin spots on the strip was used as criterion of identity. (c) When sufficient evidence was available for the existence of an intermediate, it was isolated, purified and its radioactivity determined. If the amounts present were insufficient for isolation, carrier was added, then isolated, purified by recrystallization or precipitation and its radioactivity determined. When a compound was isolated without carrier, other criteria were used such as infrared spectrum.

RESULTS

1) *Formation of cystathionine*

Both *M. edulis* and *R. cuneata* were given a solution of S^{35} -methionine (approximately 500,000 counts). After 24 hours the organisms were extracted and the extracts fractionated. Fraction B in each case was chromatographed on paper

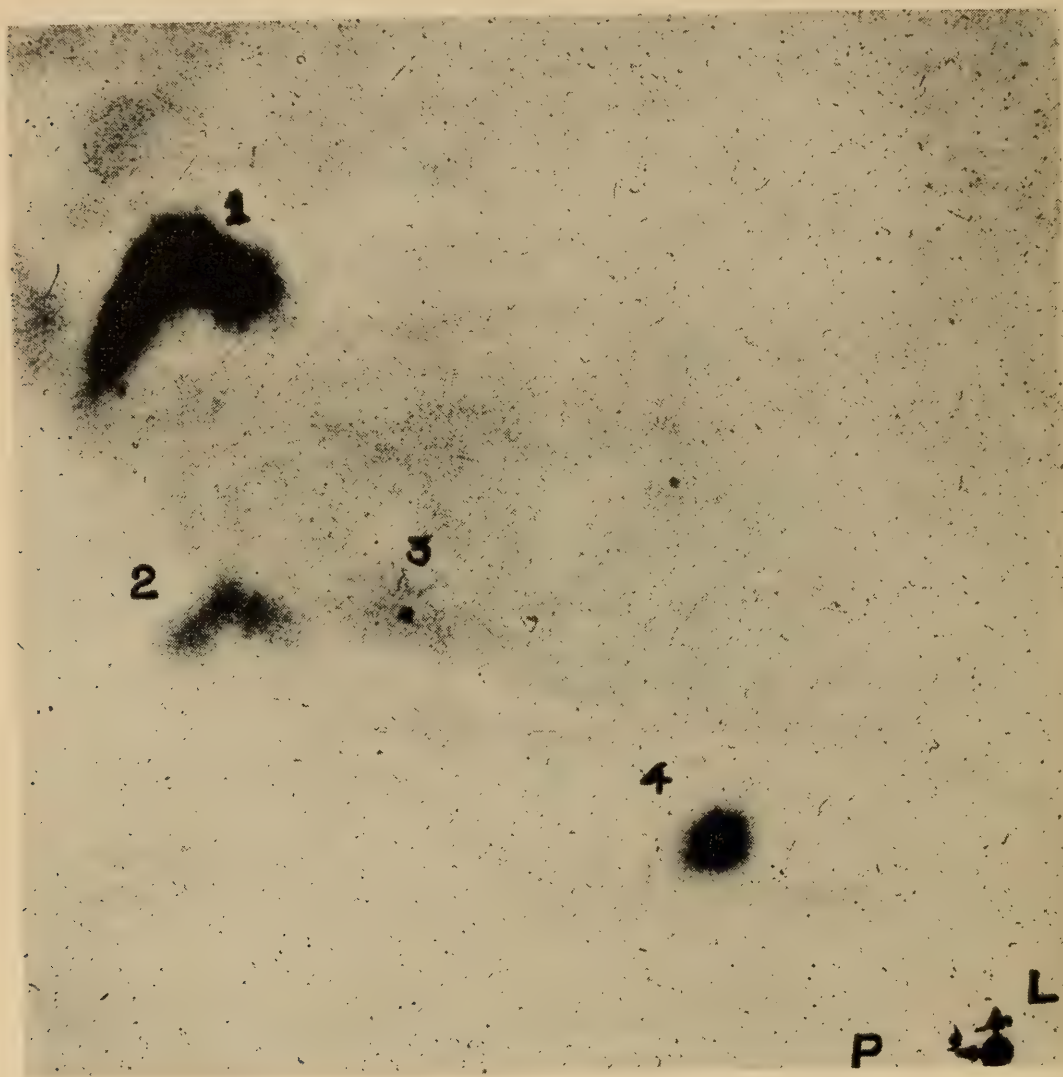


FIGURE 1. Radioautograph of fraction B from *M. edulis* after the administration of S^{35} -methionine. Solvents: phenol (P) and lutidine (L). Methionine (1), methionine sulfone ? (2), hypotaurine (3) and cystathionine (4).

according to the method used by Simpson, Allen and Awapara (1959), using phenol-water in one direction and lutidine-water in the second direction. Radioautographs of these chromatograms were made. The chromatograms from *R. cuneata* had no cystathionine. We cannot exclude its formation, for it could easily be a transient intermediate in this animal. It is very likely that it is formed, as we shall see in discussing formation of cystine. In Figure 1 is shown a radioautograph from *M. edulis*. Spot 4 is cystathionine and spot 3 is hypotaurine. Both of these

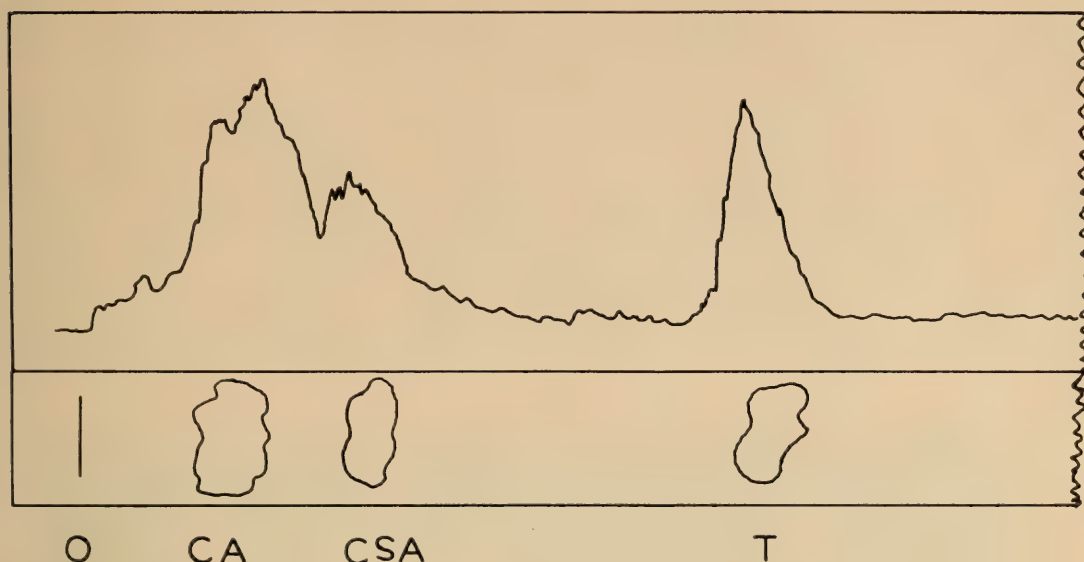


FIGURE 2. Strip chromatogram (lower) and radioactivity distribution (upper) from the acid fractions of *R. cuneata*. Solvent: phenol-water. Cysteic acid (CA), cysteine sulfinic acid (CSA) and taurine (T).

intermediates were recognized by their R_f values (cystathionine 0.27 in 72% phenol and 0.20 in 65% lutidine; hypotaurine 0.62 in 72% phenol and 0.42 in 65% lutidine). Spot 1 is methionine and spot 2, probably methionine sulfone.

2) Formation of cystine

Fraction B, from part 1, was used to identify cystine or cysteine. Neither of these amino acids can be chromatographed satisfactorily. Cystine is very insoluble and lends itself to isolation. In both instances unlabelled cystine hydrochloride was added to the fractions. Then hydrogen peroxide was added to oxidize cysteine to cystine. Cystine was precipitated from the solution by carefully adjusting the pH to 7. The crystals were filtered, washed, redissolved in dilute HCl and

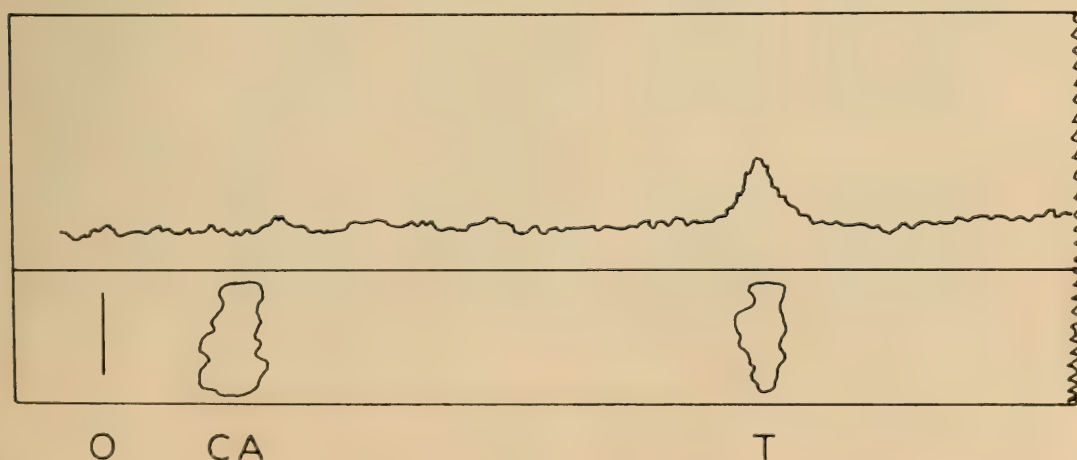


FIGURE 3. Strip chromatogram and radioactivity distribution from the acid fraction of *M. edulis*. Cysteic acid (CA) and taurine (T).

reprecipitated by the same procedure. The washed and dried crystals were used for determining radioactivity.

M. edulis

68 counts/minute/mg.

R. cuneata

120 counts/minute/mg.

Cystine or cysteine is formed in both organisms. Cysteine oxidizes readily to cystine but if one assumes that transulfuration takes place in these animals, then cystathionine and cysteine are the intermediates. Unfortunately cystathionine was not detected in *R. cuneata*. If it is not formed we must postulate another mechanism for the formation of cysteine and no other mechanism is known at this time.

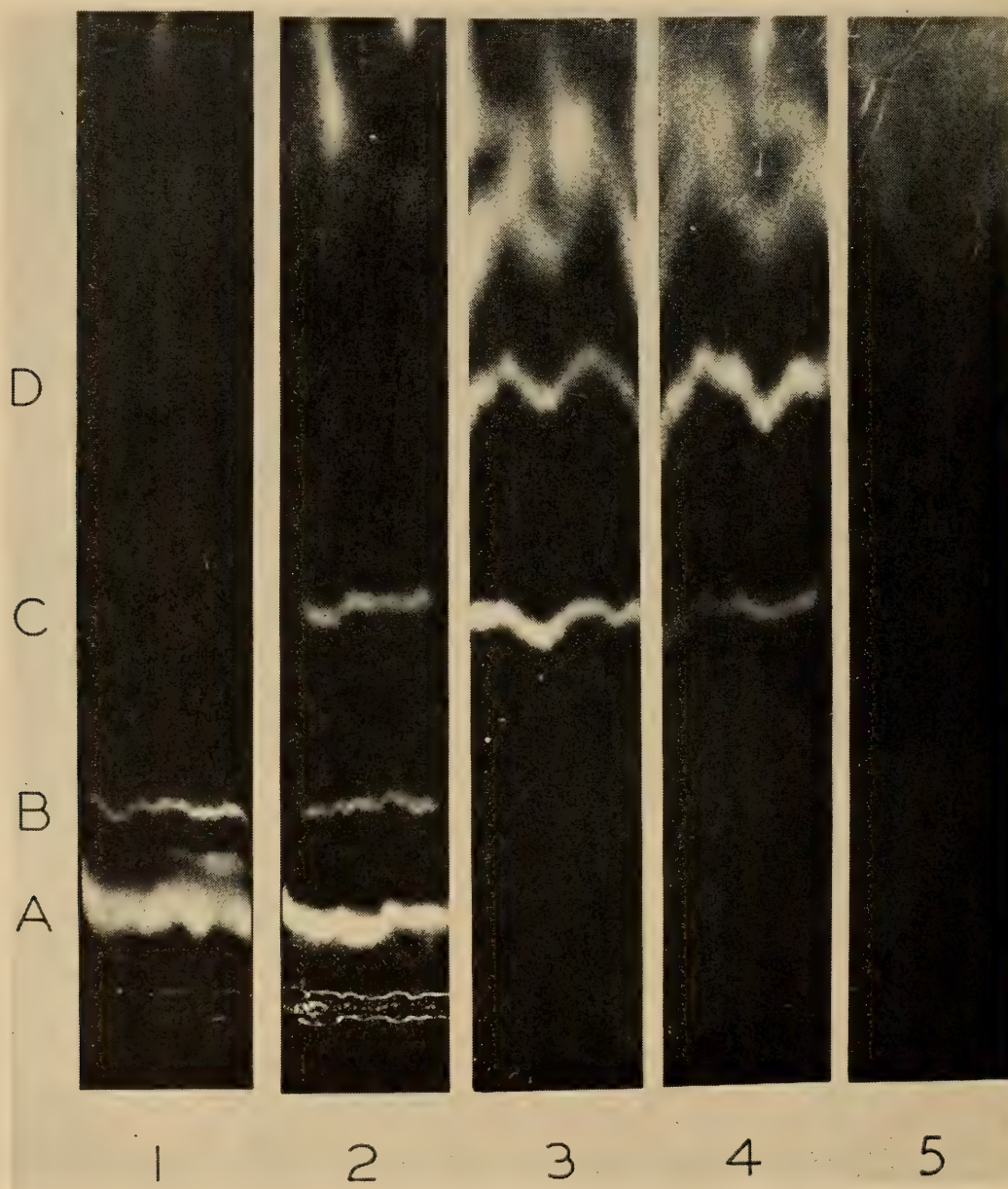


FIGURE 4. Radioautographs of the acid fraction of *R. cuneata* after the administration of methionine. (1) After 1 hour, (2) after 5 hours, (3) after 10 hours, (4) after 15 hours and (5) after 24 hours. (A) cysteine sulfinic acid, (B) cystic acid, (C) taurine, (D) unknown.

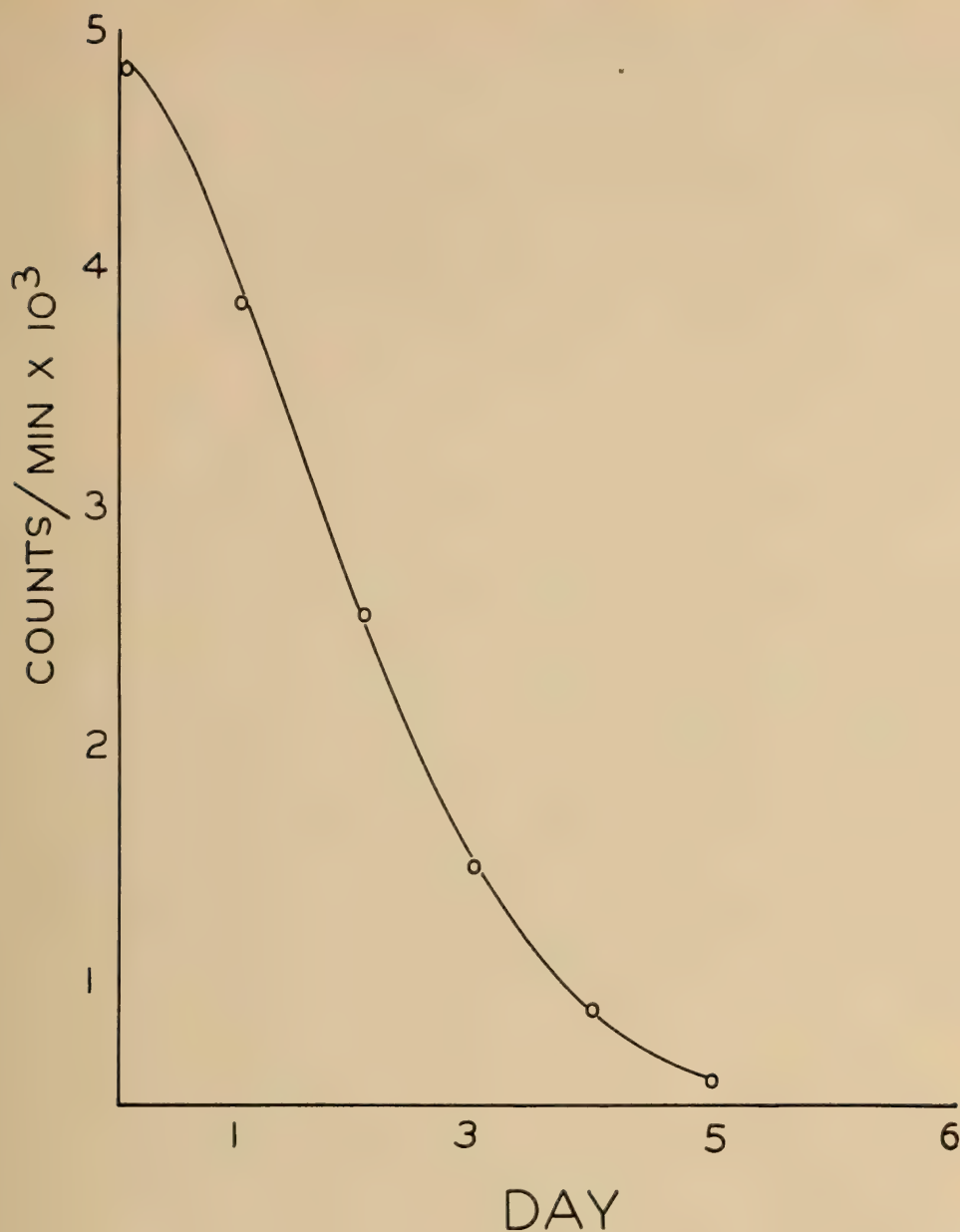


FIGURE 5. Disposition of administered S^{35} -taurine by *R. cuneata*.

3) Oxidation products of cysteine

Pooled fractions A_1 and A_2 from both organisms were mixed with cysteic acid, cysteine sulfinic acid and taurine, then chromatographed using 72% phenol. The strips were analyzed with a continuous strip counter, then developed with ninhydrin. In Figures 2 and 3 are shown the results obtained for *R. cuneata* and *M. edulis*. The latter had only one radioactive spot: taurine. Cysteic acid was formed in *R. cuneata* in relatively high amounts. A second experiment was performed on *R. cuneata* to find out the time needed for the formation of these intermediates. S^{35} -methionine was injected into several organisms; extracts were made at the end of 1, 5, 10, 15 and 24 hours. The extracts were fractionated and fraction A of each one chromatographed on strips. Radioautograms were made and they are shown in Figure 4. Taurine was formed only after cysteine sulfinic acid and

cysteic acid were formed; it took 5 hours to form sufficient taurine to be detected. At the end of 10 hours two S^{35} -labelled compounds appeared. Compound D has an R_f value identical to that of taurocyamine (.64 in phenol) but gave a negative Sakaguchi test on paper chromatograms. At the end of 24 hours most of the end products have disappeared from the animal. Taurine is formed in *R. cuneata* but not held. This is better shown in Figure 5. In this figure is shown the rate at which injected S^{35} -taurine is excreted by *R. cuneata*. One could argue that the conditions were abnormal and that the animal ejects an exogenous substance; this is not the case as seen in Figure 5; taurine is endogenously formed and it is also rapidly excreted.

4) Formation of sulfate and taurine

Both sulfate and taurine are formed by *M. edulis* and *R. cuneata*. Sulfate was detected by precipitation as $BaSO_4$ with added carrier. In both instances the $BaSO_4$ was radioactive (35 cts./min. and 85 cts./min.). Although the counts were low, they were significant. The $BaSO_4$ was ignited to insure destruction of any organic sulfur compounds.

Taurine was detected in *R. cuneata* as already shown; *M. edulis* is known to contain very large amounts of taurine (as much as 4 per cent of wet weight) and to demonstrate its formation from administered precursors would be difficult. Nevertheless we succeeded in isolating S^{35} -labelled taurine from *M. edulis* after the administration of S^{35} -methionine. The isolated taurine was recrystallized several times and its radioactivity determined. The specific activity was 27 counts/min./mg. With such low activity the evidence for taurine formation was somewhat weak. More supporting evidence was obtained by isotope dilution. To the isolated taurine we added an equal amount of pure taurine; the mixture

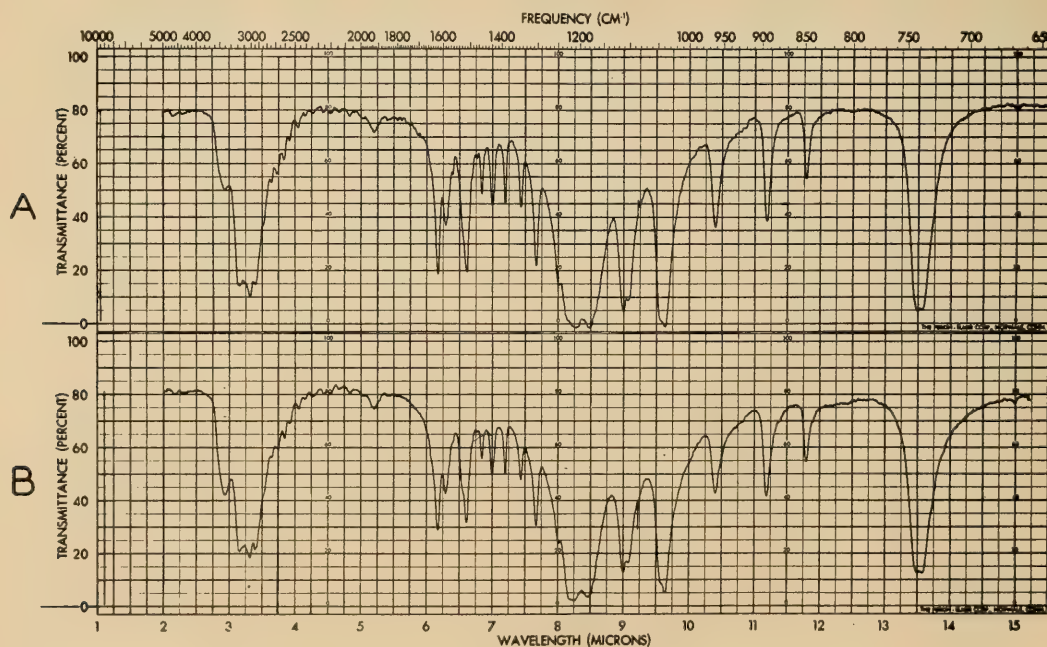


FIGURE 6. Infrared absorption spectra of (A) known taurine and (B) S^{35} -taurine isolated from *M. edulis* after the administration of S^{35} -methionine.

was recrystallized and the radioactivity of the pure crystals determined:

Isolated taurine	6 mg.	26 cts./min./mg.
Mixture	12 mg.	12 cts./min./mg.

The infrared absorption spectra³ of both pure taurine and the isolated taurine were determined and shown in Figure 6. It appears that *M. edulis* forms taurine slowly but retains it. The high concentration of endogenous taurine in this animal makes it very difficult to establish the rate at which taurine is formed.

DISCUSSION

The metabolism of sulfur amino acids in the two molluscs studied is not different, at least qualitatively, from the metabolism in mammals. The oxidation of cysteine in both, mammals and molluscs (those studied), gives rise to cysteine sulfinic acid and eventually to taurine and sulfate (Awapara, 1956). The most significant difference observed in the two animals studied is the rate at which taurine is disposed of. *M. edulis* keeps it by some unknown mechanism defying a concentration gradient. *R. cuneata* can produce taurine but cannot hold it. If we extrapolate these results, the absence of taurine in all the fresh-water animals (Simpson, Allen and Awapara, 1959) could be explained on the basis of rapid excretion, not lack of formation. The method of formation of taurine and sulfate in both organisms again appears similar to the method of formation in mammals. Cysteine sulfinic acid is either decarboxylated to hypotaurine or forms sulfate after transamination, splitting of SO₂, and oxidation. The absence of hypotaurine in *R. cuneata* and the presence of large amounts of cysteic acid is indicative of a pathway in which cysteine sulfinic acid is oxidized first to cysteic acid and this decarboxylated to taurine. In *M. edulis* hypotaurine was present but cysteic acid was not detected. If there are any differences in the metabolism of sulfur amino acids in the molluscs studied, the differences are in the intermediates and in the disposition of the end products.

The role of taurine in marine molluscs remains unknown. The only suggestion made is that it serves as an osmoregulator.

SUMMARY

1. The metabolism of sulfur-bearing amino acids was investigated in *R. cuneata* and *M. edulis*, *in vivo*.

2. Injected S³⁵-methionine gives rise to cysteine/cystine in both animals. Cystathionine was detected in *M. edulis*, but not in *R. cuneata* though it is probably formed. A demethylation and transulfuration mechanism is postulated for this conversion.

3. Both *R. cuneata* and *M. edulis* form sulfate from injected methionine. Also, both form taurine but *R. cuneata* does not accumulate it whereas *M. edulis* accumulates it in large amounts.

4. Taurine is probably formed by different reactions in these species. In *R. cuneata* it is probably formed by decarboxylation of cysteic acid, whereas in *M. edulis* it is formed by oxidation of hypotaurine.

³ We are indebted to Dr. T. Patton from the M. D. Anderson Hospital for this determination.

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THE EFFECT OF SALINITY AND TEMPERATURE ON LARVAL DEVELOPMENT OF *SESARMA CINEREUM* (BOSC) REARED IN THE LABORATORY ¹

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Although environmental factors have been known to affect the rate of development, the number of stages, and the survival of marine invertebrate larvae, relatively few studies have been made to determine the effect of a wide range of salinities, temperatures, and food on larval development under laboratory conditions. Studies on the relationship between salinity and fertilization (Amemiya, 1926), salinity and larval development (Loosanoff, 1948), and salinity and survival and growth (Davis, 1958) have been made on some mollusks. A limited number of similar investigations have also been reported on the effects of temperature (Clark, 1935; Loosanoff and Davis, 1950; and Loosanoff, Miller and Smith, 1951). While no identical studies have been carried out on other major phyla some comparable data on echinoderm development in relation to salinity and temperature may be found in the publications of Mortenson (1931, 1937 and 1938). In general, the effects of salinity, temperature, and food on the larval development of the Crustacea are not known, presumably because of the difficulties which are usually encountered in rearing larvae in the laboratory.

In spite of the persistent interest in decapod larvae and the numerous studies on life-histories reconstructed from planktonic material, only 7 accounts are known to us which deal with the complete larval development of *Brachyura* in the laboratory (Schlegel, 1911; Lebour, 1928; Hart, 1935; Sandoz and Hopkins, 1947; Chamberlain, 1957; Knudsen, 1958; and Costlow, Rees and Bookhout, 1959). None of these authors consider the effects of salinity or temperature on larval development. Broekhuysen (1937) studied the effects of temperature and salinity on egg development of *Carcinides maenas* but did not rear the larval stages. Sandoz and Rogers (1944) reported the effects of environmental factors on some of the early larval stages of *Callinectes sapidus*, reared in the laboratory, and later they conducted similar experiments on megalops obtained from planktonic material (Sandoz and Rogers, 1948). More recently, the effects of salinity and temperature on the complete larval development of *Callinectes sapidus* Rathbun have been described (Costlow and Bookhout, 1959) but mortality in this species is high within a wide range of environmental conditions and cannot be attributed solely to salinity or temperature. In the Macrura, Templeman (1936a) described the effects of temperature, light, salinity, and diet on the larvae of *Homarus americanus*, and Broad (1957) studied the relationship between food and the number of larval

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stages of *Palaemonetes vulgaris* and *P. pugio*. In the Anomura, Coffin (1958) considered the effects of salinity and temperature on the larval development of *Pagurus samuelis*.

During the past three years we have followed the larval development of a variety of Brachyura from the Beaufort area, from hatching to the first crab stage and beyond. The purpose of the present work has been to determine the effect of various salinity-temperature combinations on the number of larval stages, the molting frequency, the duration of larval life, and the survival of the larval stages of *Sesarma cinereum* (Bosc).

Inasmuch as only the first zoeal stage of *Sesarma cinereum* has been figured (Hyman, 1924) a description of the four zoeal stages and one megalops stage will be reported in a separate account.

The authors wish to thank Mrs. W. A. Chipman and Mrs. C. King for their technical assistance throughout the study. Dr. Fenner Chace, U. S. National Museum, was most helpful in his identification of the adult females, and specimens from which the larvae hatched have been deposited with the Museum.

METHODS

Ovigerous *Sesarma cinereum* (Bosc) were obtained at the drift line in the vicinity of the Duke University Marine Laboratory, Beaufort, N. C., and brought into the laboratory. The females were maintained separately at 25° and 30° C. in fingerbowls containing filtered sea water of the salinity in which the larvae were to be reared. The water, containing 200,000 units of penicillin G per liter, was changed daily. When hatching was observed, the female was removed to a separate bowl and the zoeae transferred to mass cultures of approximately 50–70 per bowl. Fertilized *Arbacia* eggs and recently hatched *Artemia* nauplii were added immediately. Zoeae were then placed in either Syracuse watch glasses (10 per watch glass) or compartmented plastic boxes (6 per compartment) containing approximately 30 ml. of water of the same salinity, temperature, and type of food as in the mass cultures. The containers were marked, covered, and placed in temperature cabinets regulated to provide a photoperiod of 12 hours of light and 12 hours of darkness. Table I gives the salinity and temperature combinations

TABLE I

Initial number of zoeae, number of megalops, and number of crabs reared under the three temperatures and four salinities indicated

p.p.t.	20° C.			25° C.			30° C.		
	Initial number	Number megalops	Number crabs	Initial number	Number megalops	Number crabs	Initial number	Number megalops	Number crabs
12.5	108	0	0	102	13	0	108	18	0
20.1	96	34	1	105	53	4	113	52	10
26.5	104	52	4	107	48	26	107	20	5
31.1	108	21	0	100	2	0	108	9	0

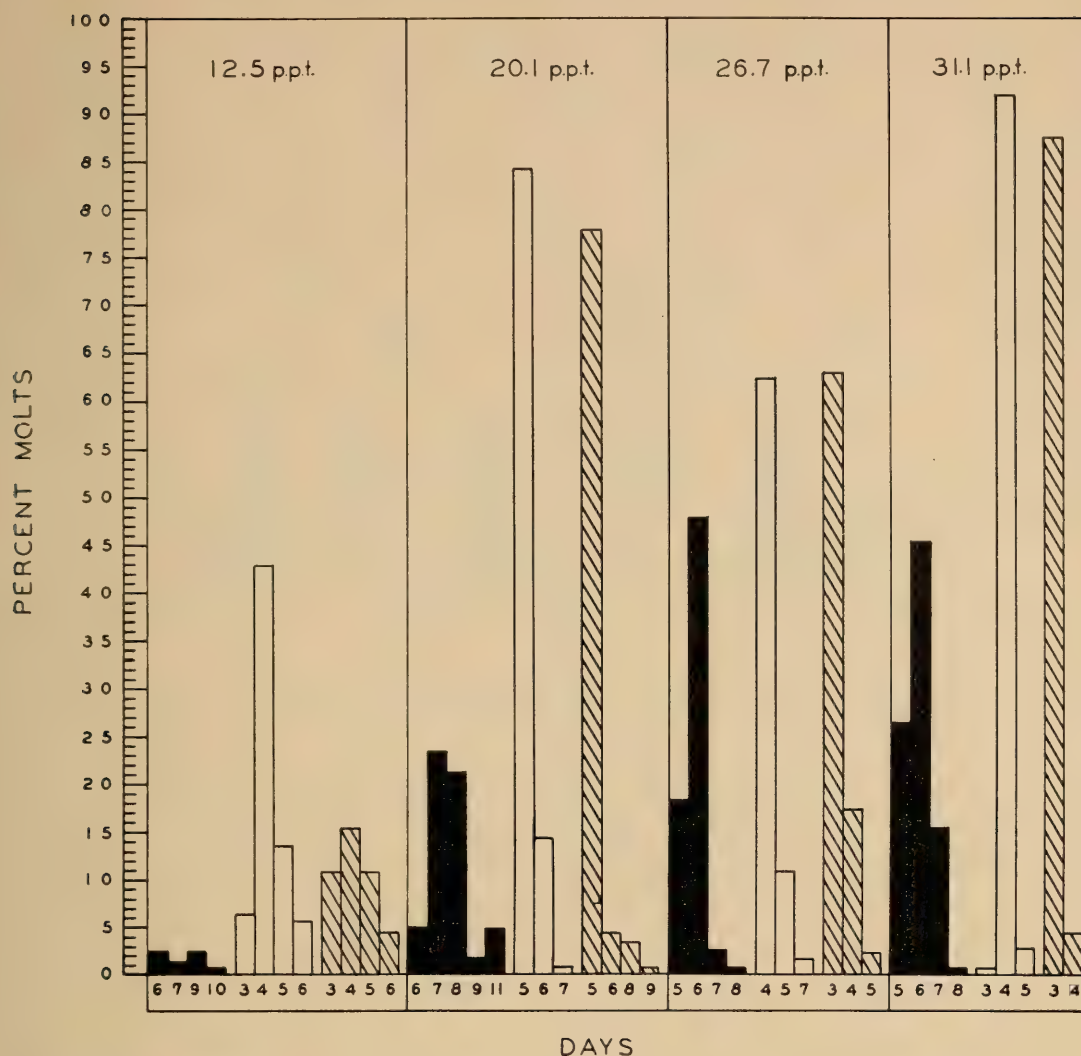


FIGURE 1. Comparison of the time of the first molt for zoeae of *Sesarma cinereum* reared in the laboratory at different salinities and temperatures. Black—20° C., white—25° C., diagonal—30° C.

used. Larvae reared at 20° C. were hatched at 25° C. and the temperature was gradually reduced.

The containers and larvae were examined daily under a dissecting microscope, the zoeae changed to freshly filtered sea water, and *Artemia* nauplii added. At this time the number of exuviae in each series was recorded and the mortality noted. When the megalops stage was reached the larvae were removed to separate bowls and fed chopped liver in addition to the *Artemia* nauplii. The first crab, and all subsequent stages, were maintained on a diet similar to that of the megalops.

RESULTS

The larvae of *S. cinereum* hatched in all salinity-temperature combinations shown in Table I. Approximately 100 per cent of the eggs hatched as first zoea, left the egg mass almost as one swarm, and swam to the light side of the bowl.

The time after hatching at which the first zoea started to molt was similar at corresponding temperatures, regardless of the variations in salinity (Fig. 1).

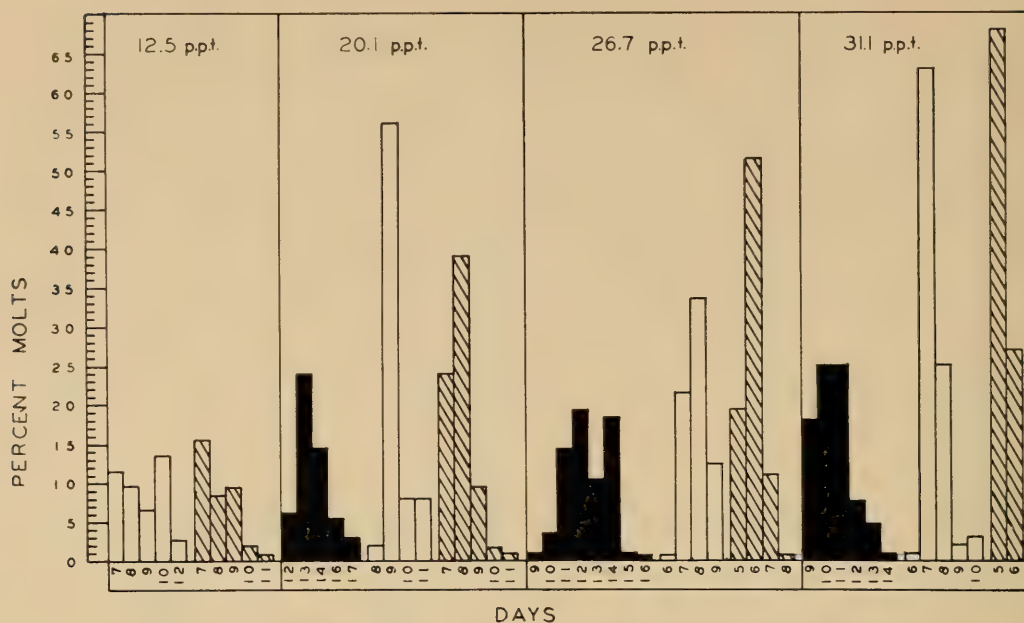


FIGURE 2. Comparison of the time of the second molt for zoeae of *Sesarma cinereum* reared at different salinities and temperatures. Black—20° C., white—25° C., diagonal—30° C.

At 26.7 and 31.1 p.p.t. the majority of the first zoeae molt within a more limited period of time than at 12.5 and 20.1 p.p.t., at the same temperature. For example, at 30° C., 31.1 p.p.t. molting was limited to days 3 and 4, with 88 per cent molting on day 3, while at 30° C., 12.5 p.p.t. the period of ecdysis was spread over four days with no pronounced peak on any one day (Fig. 1). This becomes more evident with later molts.

As shown in Figure 2, 93 per cent of the zoeae molted for the second time on days 5 and 6, at 30° C., 31.1 p.p.t.; at 30° C., 12.5 p.p.t. the zoeae did not begin the second molt until the seventh day and then required five days before all had

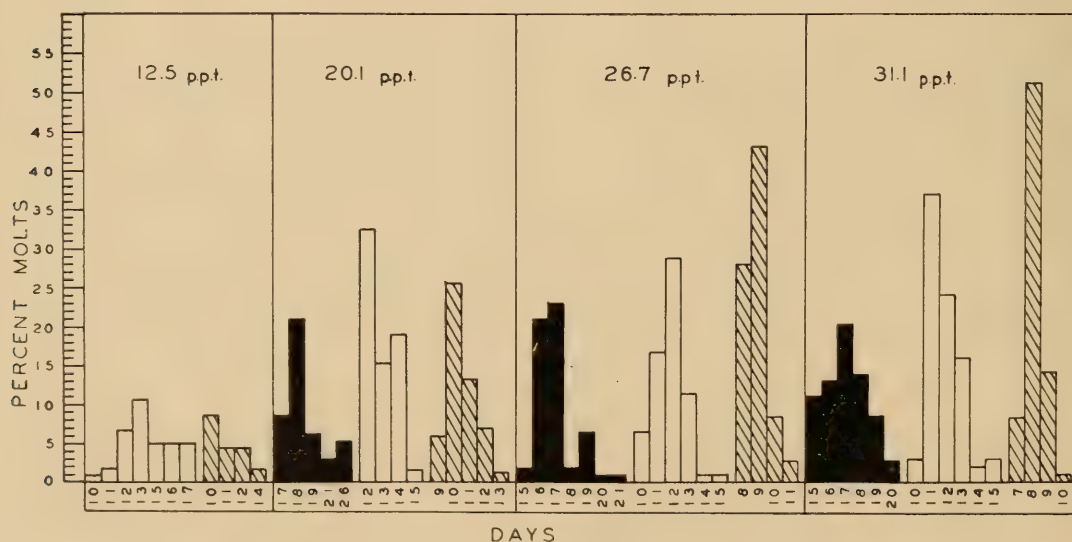


FIGURE 3. Comparison of the time of the third molt for zoeae of *Sesarma cinereum* reared at different salinities and temperatures. Black—20° C., white—25° C., diagonal—30° C.

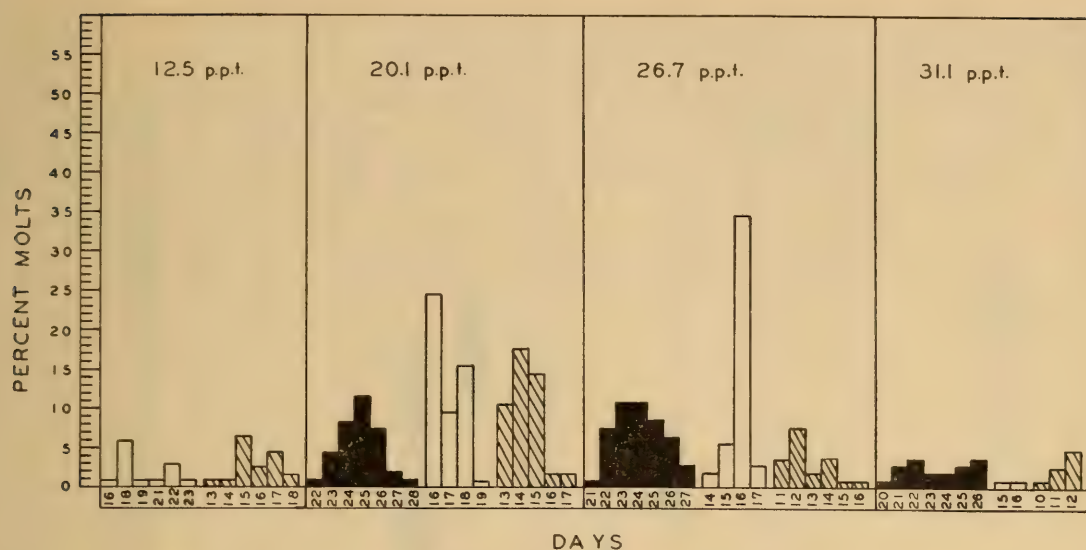


FIGURE 4. Comparison of the time of the fourth molt for zoeae of *Sesarma cinereum* reared at different salinities and temperatures. Black—20° C., white—25° C., diagonal—30° C.

completed ecdysis. The persistent lag and the tendency for each molt to require a greater period of time in the lower salinities are continued in the third and fourth zoeal molts (Figs. 3 and 4). The fourth zoeal molt produces the megalops which completes metamorphosis by molting into the first crab stage. The majority of megalops in 26.7 p.p.t. molted to the first crab stage before those in corresponding temperatures at 20.1 p.p.t. (Fig. 5). Megalops at other salinities, 12.5 and 31.1 p.p.t., did not survive to the first crab stage.

The duration of the four zoeal stages, the megalops stage, and the total time required for development to the first crab stage are affected by salinity as shown in Figure 6. There is a gradual reduction in the times required from low to high salinity. The shortest time for zoeal development was 10 to 12 days, at 30° C., 31.1 p.p.t., while at 30° C., 12.5 p.p.t. the same development required 13 to 18 days. Similar differences are shown for the other temperatures used (Fig. 6). Variations in the time of megalops development are also associated with salinity. At 30° C., 26.7 p.p.t. the duration of the megalops stage was 7 to 8 days while at 30° C., 20.1 p.p.t. the megalops persisted for 10 to 16 days (Fig. 6). Since the

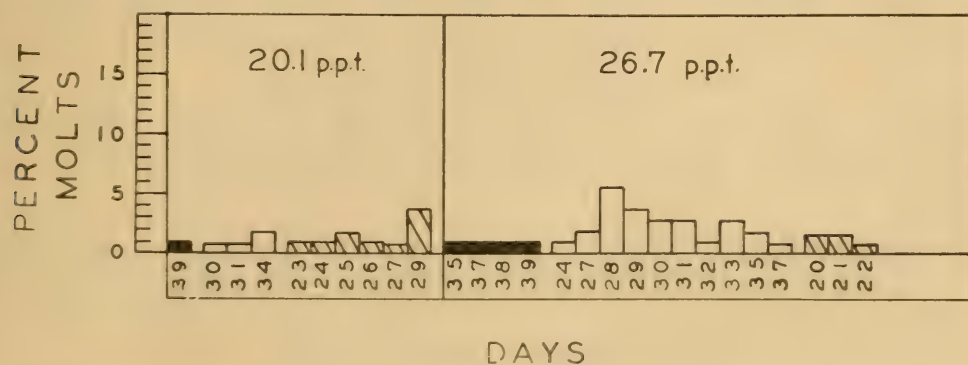


FIGURE 5. Comparison of the time of the molt from the megalops to the first crab stage of *Sesarma cinereum* reared at different salinities and temperatures. Black—20° C., white—25° C., diagonal—30° C.

zoal and megalops stages are prolonged in lower salinities, the time required for complete development to the crab is delayed (Fig. 6).

As might be expected, the first molt occurred latest at 20° C. and earliest at 30° C. (Fig. 1). The time for completion of the second molt is spread over a greater number of days at 20° C. and by the time of the third molt (Fig. 3), the

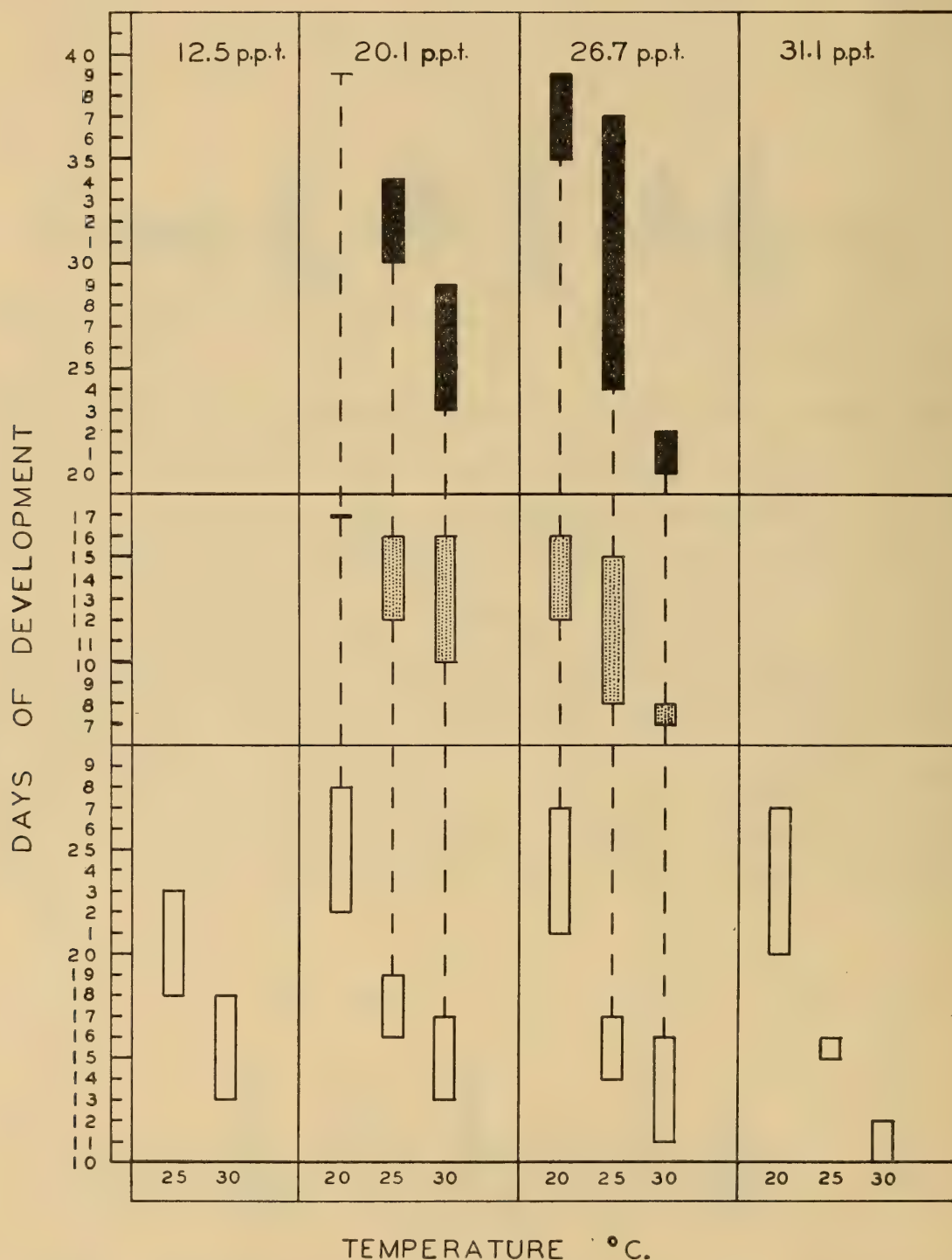


FIGURE 6. Comparison of minimum and maximum time of zoeal development (white), megalops development (stippled), and total development (black) to the first crab stage for larvae of *Sesarma cinereum* reared at different salinities and temperatures.

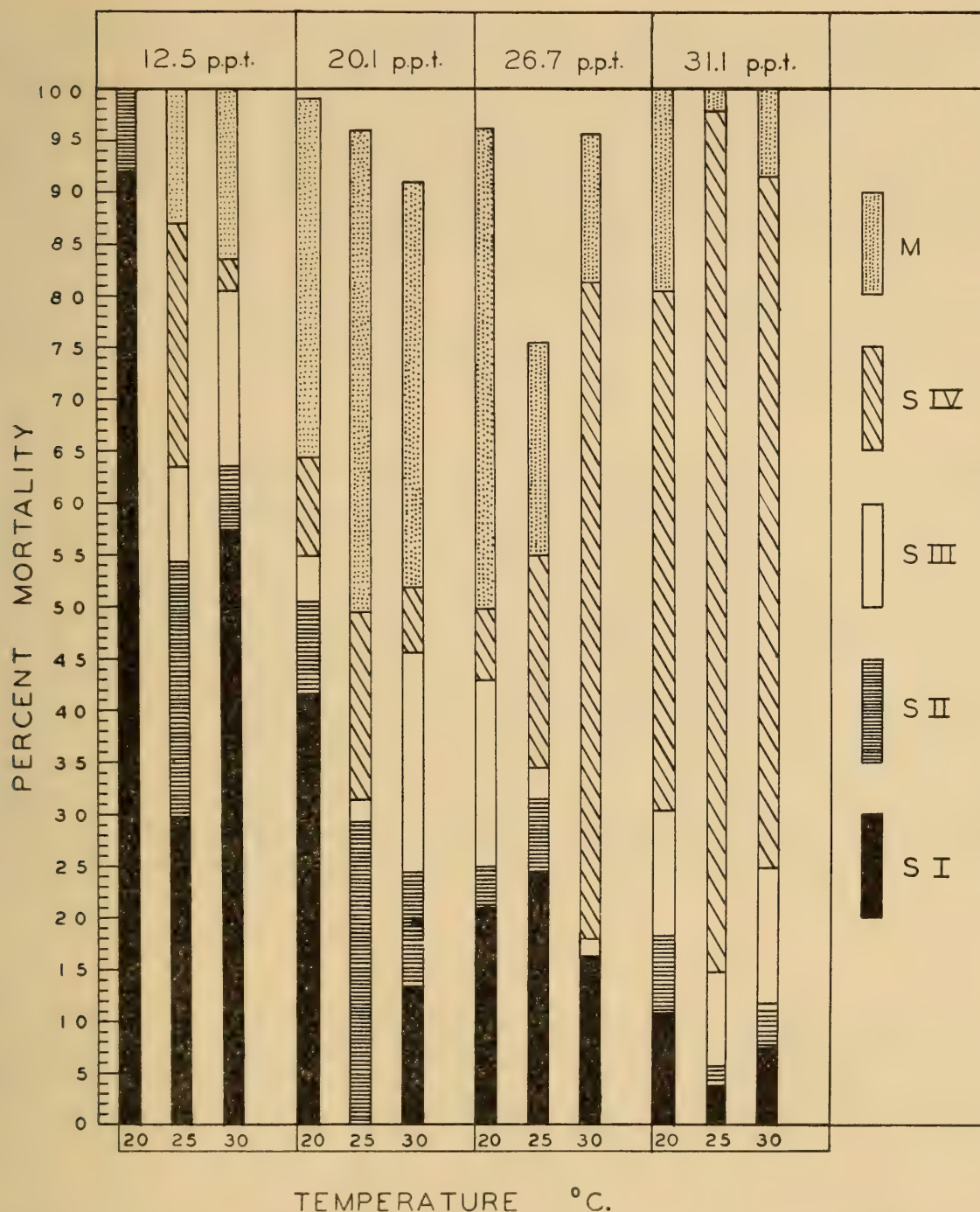


FIGURE 7. Per cent mortality of each stage of *Sesarma cinereum* reared at different temperatures and salinities. S I-S IV = zoeal stages I-IV; M = megalops.

differences due to temperature appear to be even more distinct. At 30° C., the majority of second stage zoeae have completed the third molt by the time the zoeae at 25° C. have begun this molt. Similarly, the zoeae at 25° C. have completed the third molt before those at 20° C. have begun. At molt IV (Fig. 4) there is an even greater difference in zoeae reared at different temperatures and at 20° C. the spread of time for the molt is considerable. This difference is carried over into the molt to the first crab (Fig. 5). As shown in Figure 6, the duration of

the total larval life (*i.e.*, first zoea to first crab stage) varies considerably. The shortest time (20–22 days) required for complete development to the crab stage is found at 30° C. and the longest time (35–39 days) was for larvae maintained at 20° C. More larvae completed development at 25° C., 26.7 p.p.t. than at 25° C., 20.1 p.p.t. but the former required a greater range of time (24–37 days) than the latter (30–34 days). A Q_{10} of approximately 1.8 was observed for the time required for complete development to the first crab stage at 20° C. and 30° C.

Survival of zoeal and megalops stages varied in relation to salinity and temperature. Figure 7 indicates that mortality of the first zoeal stage was highest at all temperatures within a salinity of 12.5 p.p.t. and at 20° C., 20.1 p.p.t. Mortality of the first stage was low (4–11 per cent) at all temperatures in a salinity of 31.1 p.p.t. The second stage zoea showed a high mortality at 12.5 p.p.t. and 20.1 p.p.t. at 25° C. In all other temperature-salinity combinations mortality of the second stage was under 10 per cent. Mortality of the third stage zoea varied in all salinities and temperatures: from 1 per cent at 30° C., 26.7 p.p.t. to 21 per cent at 30° C., 20.1 p.p.t. Mortality of stage four zoea considerably depleted the number of larvae at all temperatures at 31.1 p.p.t. (Fig. 7). The majority of the fourth stage zoeae died at this salinity in an attempt to molt to the megalops stage. The number of fourth stage zoeae which successfully molted to the megalops stage was largest at all temperatures in 20.1 p.p.t. and 26.7 p.p.t. This varied from 35 to 50 per cent of the original number of zoeae. Those series with the largest number of megalops were also those which showed some survival to the first crab stage and beyond. In these same series, however, much of the total mortality was due to death in the megalops stage. For example, while the lowest per cent mortality (49.8 per cent) of all zoeal stages occurred at 20° C., 26.7 p.p.t., the highest mortality of the megalops stage was observed at this same temperature and salinity. The highest per cent survival through the first crab stage was 24.4 per cent, at 25° C., 26.7 p.p.t.

DISCUSSION

The few successful rearing experiments with *Brachyura* larvae usually have had two major features in common: water which was limited in its temperature range and salinities which fluctuated only slightly. Experimental studies on the effects of salinity and temperature variation on decapod larvae are usually confined to a portion of the larval development and do not include all the stages prior to metamorphosis to the first post-larval stage. Sandoz and Rogers (1944) studied the first three zoeal stages of *Callinectes sapidus* but actually give results for only the first stage. A later study (Sandoz and Rogers, 1948) centered around the effects of temperature and salinity on the megalops of *C. sapidus* obtained from planktonic material. Coffin (1958) followed the development of *Pagurus samuelis* in the laboratory at two different temperatures and a variety of chlorinities. In the Macrura, several authors have made experimental studies on larval development. Sollaud (1919), rearing the larvae of *Palaemonetes varians microgenitor* at two different temperatures and salinities, found that at 15° C., 6.5 p.p.t. the larvae completed development with 6 to 8 molts in 23–33 days while at 18° C., 6.5 p.p.t. only 5 or 6 molts were required and development was complete after 15–23 days. At 15° C., “sea water,” larval development occurred in from 20 to

29 days with 5, 6, or 7 molts (Sollaud, 1919). Gompel and Legendre (1927) found that the first and second stages of *Homarus vulgaris* could withstand a salinity of 44 (1.033) but died at 23.3 (1.017). Their work does not include attempts to rear the larvae through successive molts and no reference is made to feeding. Templeman (1936a) studied the effects of a variety of environmental factors on larval development of *H. americanus*, and Broad (1957) followed the relationship between diet and number of larval stages of *Palaemonetes pugio* and *Palaemonetes vulgaris*.

In the present study three temperatures were combined with four salinities to provide twelve distinct environments. Food was always abundant in all environments, and the photoperiod was constant.

Hatching

Within the temperatures and salinities used there was little difference in the hatching process. Hatching was 100 per cent in all conditions and the so-called "pre-zoea" was never observed. Immediately after the zoeae left the egg mass some were removed, examined under a microscope, and were found to possess a dorsal spine and exposed maxillary hairs. Many authors have reported the "pre-zoea" from a variety of crabs hatched in the laboratory: Birge (1883), *Panopeus sayi*; Hyman (1925), *Eurypanopeus depressus*, *Hexapanopeus angustifrons*, *Menippe mercenaria*, *Neopanope texana sayi*, and *Panopeus herbstii*; Lebour (1928), all British species she described; Churchill (1942), *Callinectes sapidus*; and Chamberlain (1957), *Neopanopeus texana sayi*. As early as 1903 Williamson noted in experiments with *Carcinus maenas* that normal hatching produced only first zoeae; if the egg mass were washed immediately prior to hatching, the "pre-zoea" could be obtained. Sandoz and Rogers (1944) observed that the number of "pre-zoea" of *C. sapidus* at hatching was associated with salinity. At 25 to 30 p.p.t. few "pre-zoeae" were obtained while at 10.9 p.p.t., 90 to 100 per cent of the larvae hatched as "pre-zoeae" and apparently never molted to the first zoeal stage.

Sandoz and Rogers (1944) also found that the few zoeae which did hatch at 14 p.p.t. were sluggish and did not show the characteristic positive phototactic response. At 18 p.p.t. the zoeae were active and positively phototropic and at 24 p.p.t. the larvae were quite active (Sandoz and Rogers, 1944). Broekhuysen (1937) found that development of the eggs of *Carcinides maenas* was normal and that hatching occurred at 28.3–40 p.p.t., 10° C., and at 18–40 p.p.t., 15.6° C. He suggests that the range of salinity in which the crab can develop is shifted toward the more saline waters and further comments that salinity and temperature together determine the limits for development. In the present study the first zoeae of *S. cinereum* were active in most salinities and always positively phototactic. At 12.5 p.p.t. the zoeae were not as active as in the higher salinities but did seek the lighted side of the bowl.

To assure acclimation of the eggs to both temperature and salinity, the ovigerous females of *S. cinereum* were placed in the salinities to be used several days prior to hatching. Templeman (1936a) used larvae obtained from one egg mass, which were hatched at one salinity and temperature, and transferred them to the experimental salinities approximately two hours after hatching. He does not indicate

whether the change was gradual or abrupt. Sandoz and Rogers (1944) apparently followed a similar approach with some of the first zoeae of *C. sapidus* but allowed 24 hours before transferring them to the different salinities. A salinity range of 23 to 28 p.p.t. was found to be optimum for the hatching of zoeae of *C. sapidus* (Sandoz and Rogers, 1944). Coffin (1958) hatched the eggs of *Pagurus samuelis* at one salinity and temperature and immediately transferred the zoeae to the experimental conditions (personal communication). In these three studies, however, the best results were obtained with zoeae in salinities similar to the water in which the eggs were hatched. Thus, one questions whether the salinities described as "optimum" might not be due to the fact that larvae reared in water of the salinity at which they were hatched were not required to acclimate to abrupt salinity changes. In the present study separate crabs were used for each salinity to avoid this disadvantage. This procedure may, however, introduce a variable which is difficult to recognize or control: differences in viability of larvae obtained from different females. It is thought that this factor may account in part for the slight differences in molting frequency and duration of total larval life in larvae reared in 20.1 p.p.t.

Larval development

In the present study there is definite evidence that the duration of the individual stages and the over-all time required for complete development were affected by the salinities used. In the lower salinities, 12.5 and 20.1 p.p.t., there was a tendency for molting of the individual stages to begin later than at the higher salinities. While this may be seen for each stage in Figures 1-5, it is best shown in the total time required for zoeal development (Fig. 6). At comparable temperatures, there is a gradual reduction in the time of development from low salinity to high salinity. Templeman (1936a) found that the larvae of *H. americanus* required approximately the same number of days to develop to stage IV in salinities of 21.8 to 31.8 p.p.t. He suggests that molting may occur earlier at 21 p.p.t. and 25 p.p.t. but the small number of larvae (12) used at each salinity do not give conclusive evidence. Templeman (1936a) also found that as the salinity was reduced, the number of first stage larvae which molted to the second stage also decreased: at 11.6 p.p.t. none molted to the second stage and in higher salinities, 42.5 p.p.t., the results were similar. The results of Sandoz and Rogers (1944) with zoeae of *C. sapidus* indicate that the duration of the first stage is similar in both 20 and 25 p.p.t., at comparable temperatures. In salinities below 18 p.p.t. and above 29 p.p.t. the activity of the larvae decreased. A similar analysis of the duration of stages II and III is not given. In a later study Sandoz and Rogers (1948) followed the time required for megalops of *C. sapidus* to molt to the first crab stage in different salinities and in two rather broad temperature ranges. As the salinity decreased, from 31 to 10 p.p.t., a greater length of time was required for completion of the final larval molt (Sandoz and Rogers, 1948). At 11-17° C., the increase in time, with the same decrease in salinity, is even more obvious but as existing light conditions varied, the two experiments should not be compared directly. Inasmuch as the megalops were obtained from the plankton, and the age of the individuals was not definitely known, it is difficult to evaluate the significance of the 17 hours difference in molting. Coffin (1958) shows the dura-

tion of each stage of *Pagurus samuelis* at several chlorinities. With the exception of the first zoeae, the duration of the stages was less at a chlorinity of 16 (sal. 28.9 p.p.t.) than at 19.3 (sal. 34.9 p.p.t.). This comparison includes the first three zoeal stages only (Coffin, 1958). Sollaud (1919) notes that a decrease in salinity, presumably from approximately 35 p.p.t. ("sea water") to 6.5 p.p.t., increases the time required for complete larval development of *Palaemonetes varians microgenitor*. In the present study, a decrease in salinity prolonged the length of zoeal development of *S. cinereum*. Although the time required for development of *S. cinereum* megalops to the first crab stage follows this trend, it is not as definite as the results for the zoeae and there is more overlapping in salinities of 20.1 and 26.7 p.p.t. (Fig. 6).

Survival of *S. cinereum* zoeae was definitely affected by salinity. As shown in Figure 7, the first stage zoea withstood the higher salinities (26.7–31.1 p.p.t.) better than the lower salinities (12.5 p.p.t.–20.1 p.p.t.). Mortality in the second and third stages was relatively low in all salinities. While exact comparisons in stage cannot be made, it should be noted that mortality in *Homarus americanus* (Templeman, 1936a) was highest in the third and fourth stages in the lower salinities. Mortality of first zoeae of *Pagurus samuelis* was lowest at a chlorinity of 19.3 (sal. 34.9 p.p.t.), the chlorinity at which the eggs had been hatched (Coffin, 1958). In the remaining larval stages, the per cent mortality was similar in both 16 and 19.3 (sal. 28.9 p.p.t. and 34.9 p.p.t.). Mortality of the fourth stage of *S. cinereum* varied from 50–83 per cent of the total mortality in 31.1 p.p.t. In the vast majority of cases the fourth stage zoea died in the process of molting into the megalops. Those larvae which did manage to successfully complete the fourth molt usually died within a few days.

The effect of temperature on the duration of the individual larval stage of *S. cinereum* is shown in Figures 1 through 5. With the exception of the zoeae reared at 20.1 p.p.t., duration of the individual stages at 30° C. was approximately half the time required for comparable development at 20° C. At 25° C. there was considerable overlapping in the time of development. In general there is a greater range of time for all molts at 20° C. than at 30° C. although an insignificant number of late molts at the latter temperature occasionally spread the over-all time. Templeman (1936a) did not consider the effect of temperature on duration of the individual stages, except indirectly and with light as an additional variable. He does give, however, the time for development to the fourth stage of *Homarus americanus* reared at different temperatures. He found that at 24° C., 10.5 days were required while at 14° C., 26.5 days were required for comparable development. Sandoz and Rogers (1944), while not giving similar figures for *C. sapidus*, do note that below 20° C. none of the first zoeae completed the molt into the second stage. Sandoz and Rogers (1944) also state that when other factors were favorable, active feeding occurred at temperatures of 19° to 23° C. The effect of temperature on the time required for completion of the four zoeal stages and the megalops stage of *S. cinereum* is shown in Figure 6. There is some overlapping at 25° C. but the minimum and maximum at 20° C. and 30° C. are quite discrete.

Broad (1957) found that the number of stages of *Palaemonetes pugio* and *Palaemonetes vulgaris* varied according to the diet, and Templeman (1936a, 1936b) found that a reduction in food produced a longer intermolt period and

sometimes produced an "extra" larval stage. In the present study a super-abundance of food was present in all 12 environments and variations in number of larval stages were never observed. The four zoeal stages and one megalops were found to be consistent throughout the study of 1266 larvae. Thus, while variations in diet may account for additional stages in the laboratory, variations in temperature and salinity do not alter the number of stages of *S. cinereum*.

Ecological significance

S. cinereum, ranging from the Chesapeake Bay to Mexico (Rathbun, 1917), is found at Beaufort, N. C., in areas of varying salinity. Pearse (1936), during the summer of 1928, found adult crabs adjacent to waters varying in salinity from 0.5 to 18.9 p.p.t. During the summer of 1958 and 1959 the water in the collecting areas was 26.5 to 35 p.p.t. Although the limits of the breeding season are not known at Beaufort, N. C., ovigerous females of *S. cinereum* are abundant during the summer months and Sutcliffe (1950) found the greatest numbers of decapod larvae in the plankton at Beaufort throughout these months. In the laboratory, hatching was successful in salinities of 12.5 to 31.1 p.p.t. and it may be assumed that hatching of the larvae takes place naturally in waters where there is a wide salinity range. The time required for complete larval development of *S. cinereum* in the natural environment is not known. Because of differences in species, temperature, and food it is impossible to directly compare our results with the findings of other rearing studies. However, it may be noted that Sandoz and Hopkins (1947) found that the four zoeal stages and one megalops stage of *Pinnotheres ostreum* required 25 days, at 23° C., for complete development. Chamberlain (1957), rearing *Neopanope texana sayi* at 24° C., reported that development of the same number of stages required 20–25 days. Hart (1935), while commenting that the length of larval life is dependent on a number of environmental factors, gives four weeks for the two zoeal stages and one megalops of *Pinnotheres taylora*, but does not mention the temperature or salinity used in the laboratory rearing. At 25° C. *Sesarma cinereum* required 24–37 days and at 30° C., 20 to 22 days (Fig. 6).

While it is beyond the scope of this study to discuss the theories of plankton retention or the effects of tidal currents on zooplankton, some of our results may be compared with the findings of some recent field studies. Bousfield's (1955) excellent study on barnacle larvae in the Miramichi Estuary also includes some references to decapod larvae. He did not find the larvae of *Rhithropanopeus harrissi* in salinities higher than 23.5 p.p.t. but did find them in salinities as low as 4 p.p.t. Connolly (1925) also notes that larvae of this same species are found in brackish to fresh water but does not give salinities. Bousfield (1955) states that salinity is probably the chief factor affecting survival of barnacle larvae in the upriver limits of the estuary. Although not identified, either by species or stage, Sutcliffe (1950) found the largest number of decapod larvae in waters of high salinity (31.0–35.4 p.p.t.) and in temperatures of 24.9–30.0° C. He did not, however, investigate a variety of depths. It has been suggested from field studies that the survival of larvae of estuarine forms is associated with salinity and temperature and, as indicated by Bousfield (1955), also associated with vertical distribution and horizontal water movements. In the laboratory (Fig. 7) 100 per

cent mortality occurred at low salinities (12.5 p.p.t.) and at high salinities (31.1 p.p.t.), whereas in the intermediate salinities (20.1 and 26.7 p.p.t.) some zoeae passed through all larval stages and metamorphosed to the first crab stage at all three temperatures. The constant conditions of temperature and salinity, used in the laboratory studies, are obviously not found in the natural estuarine environments. If zoeae were hatched and retained in water of low or high salinities in nature, they might not survive. If, however, the vertical and horizontal water movements carried them to intermediate salinities, their chances of survival would be increased. An examination of Figure 7 suggests that optimum conditions of temperature and salinity exist for each larval stage of *S. cinereum*.

Estimation of optimum conditions

The salinity-temperature ranges for each larval stage of *S. cinereum* have been determined from laboratory-reared material. If these same environmental limits

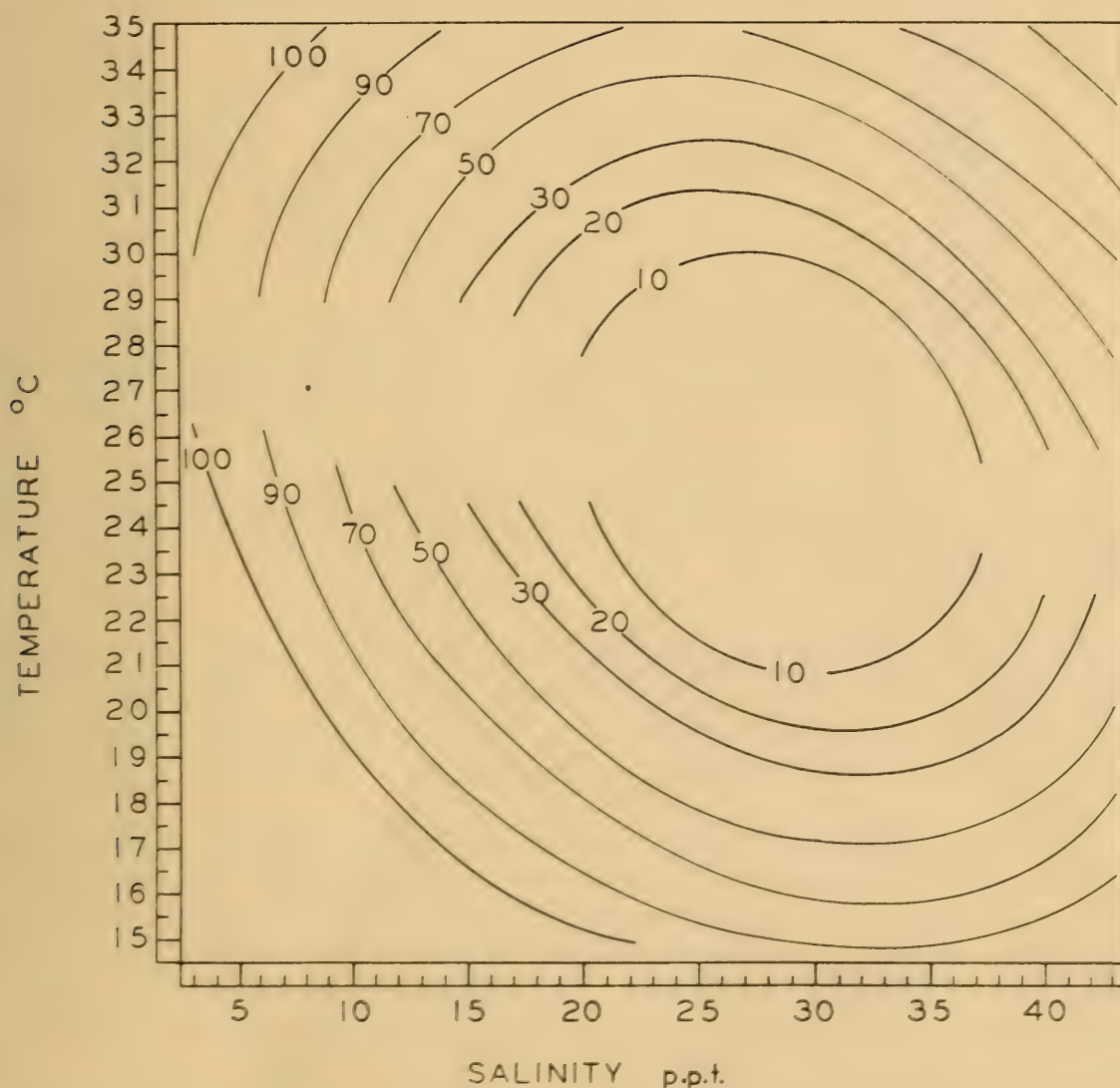


FIGURE 8. Estimation of per cent mortality of first zoeal stage of *Sesarma cinereum* based on the fitted response surface to observed mortality under twelve different combinations of salinity and temperature.

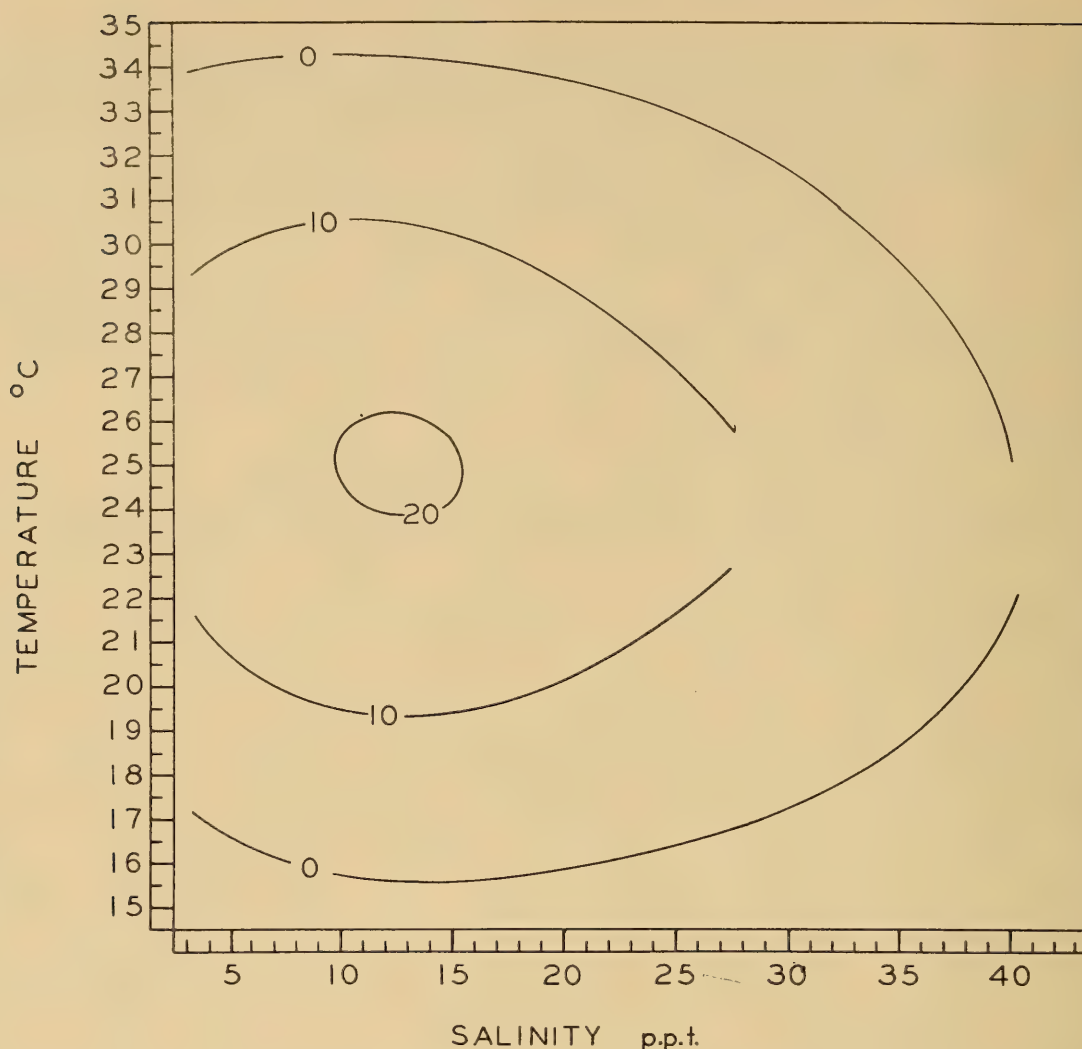


FIGURE 9. Estimation of per cent mortality of second zoeal stage of *Sesarma cinereum* based on the fitted response surface to observed mortality under twelve different combinations of salinity and temperature.

prevail in nature, one may make an estimation of the distribution and mortality of larval stages of this species in the natural environment. By using statistical methods it is possible to postulate the response of the larvae under a greater variety of environmental conditions than is possible from direct observations in the laboratory.

The basic experimental design used in this study is usually referred to as a factorial design. Specifically the plan was a 3×4 factorial using three levels of temperature and 4 levels of salinity, making in all 12 combinations of experimental conditions. If it is reasonable to assume that the effects of temperature and salinity are continuous, and further, that a temperature-salinity interaction may exist, then the 12 experimental combinations may be regarded as a sample in a continuum of temperature and salinity. Proceeding further one may postulate the existence of a continuous response (*i.e.*, per cent mortality) as a function of temperature and salinity, and that there may exist either unique optimum combinations of the two factors or that several combinations of temperature and salinity

may produce the same response. The estimation of this functional relationship has come to be called "the fitting of a response surface" (Box and Youle, 1955).

For the present study the surface fitted is described by the quadratic form

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2$$

where $y = \arcsin \sqrt{\text{per cent mortality}}$, $x_1 = \text{temperature in } ^\circ\text{C.}$, $x_2 = \text{salinity in p.p.t.}$ Then $b_0 = \text{constant}$, $b_1 = \text{linear effect of temperature}$, $b_2 = \text{linear effect of salinity}$, $b_{11} = \text{quadratic effect of temperature}$, $b_{22} = \text{quadratic effect of salinity}$, $b_{12} = \text{interaction effect between temperature and salinity}$.

The b 's were calculated from the experimental points and the observed mortality by the method of least squares. A separate equation was obtained for each stage. The contours of the surface for several selected mortality percentages were then calculated and plotted in Figures 8, 9, 10, 11, and 12.

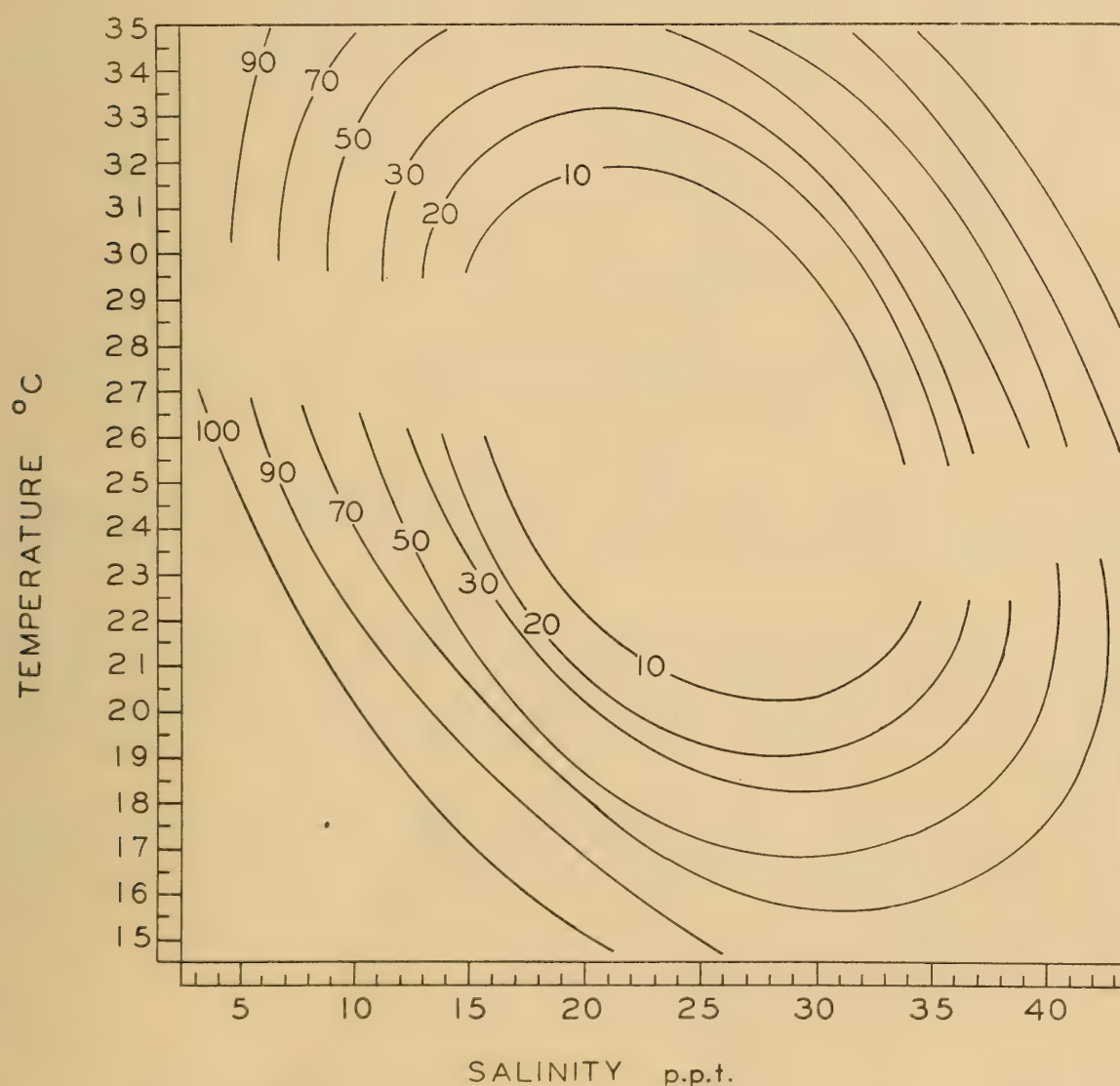


FIGURE 10. Estimation of per cent mortality of third zoeal stage of *Sesarma cinereum* based on the fitted response surface to observed mortality under twelve different combinations of salinity and temperature.

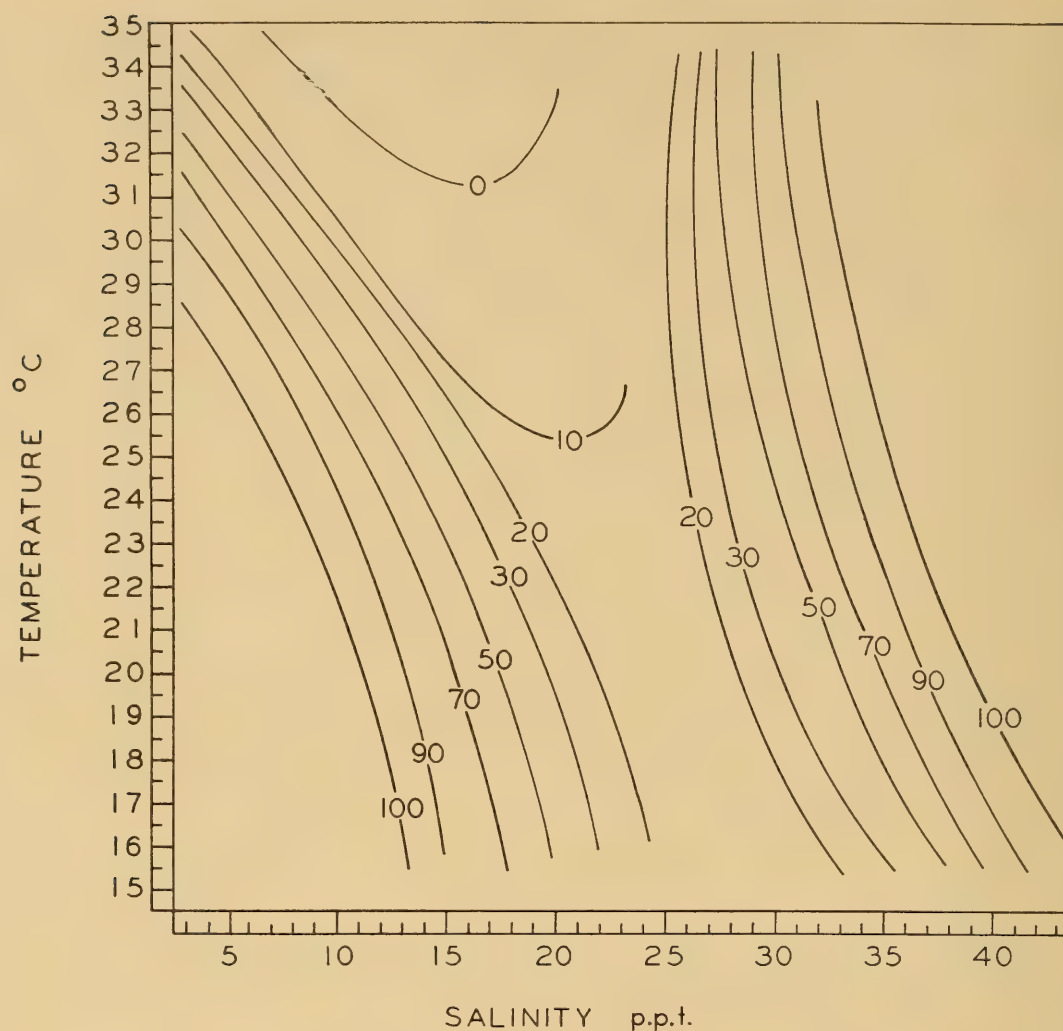


FIGURE 11. Estimation of per cent mortality of fourth zoeal stage of *Sesarma cinereum* based on the fitted response surface to observed mortality under twelve different combinations of salinity and temperature.

The contours in Figures 8 through 12 represent the predicted values of temperature and salinity which would produce the mortality indicated by the contour line. It should be noted that the usual dangers of extrapolation beyond the ranges of observed data are as inherent in this method of prediction as in any other.

Two other uses may be made of the statistics computed in the fitting process: (1) an estimated optimum combination, if one exists; and (2) tests of significance of individual effects using the residual error mean square as an estimate of the experimental error.

The following maximum or minimum conditions were observed:

	Temp. °C.	Salinity, p.p.t.	
Stage I	25.5	27.9	min. mortality = 1.9%
Stage II	25.0	12.4	max. mortality = 20.4%
Stage III	26.0	24.1	min. mortality = 3.5%
Stage IV	No maximum or minimum		
Stage V	No maximum or minimum		

In making individual tests of significance the significance levels chosen were 5 per cent, 10 per cent, and 20 per cent since the exploratory nature of this study was to identify possible leads toward important factors or combination of factors. Effects testing significance at the 5 per cent level were denoted as "marked effects," those testing at the 10 per cent and 20 per cent level as "some effects." T and T^2 were used to denote, respectively, the linear and quadratic effects of temperature, S and S^2 the same for salinity and $(T \times S)$, the interaction. A summary of these tests follows:

	"Marked effects"	"Some effects"	"Over-all correlation"
Stage I	S	T, T^2	0.872
Stage II		S, T^2	0.742
Stage III		$T, T^2, S, (T \times S)$	0.829
Stage IV	$S^2, (T \times S)$	S	0.877
Stage V	S	$(T \times S)$	0.847

It is worth noting that the interaction effects which begin to show up in the third stage are reflected in the contours in Figures 8 through 12. Stages I and II

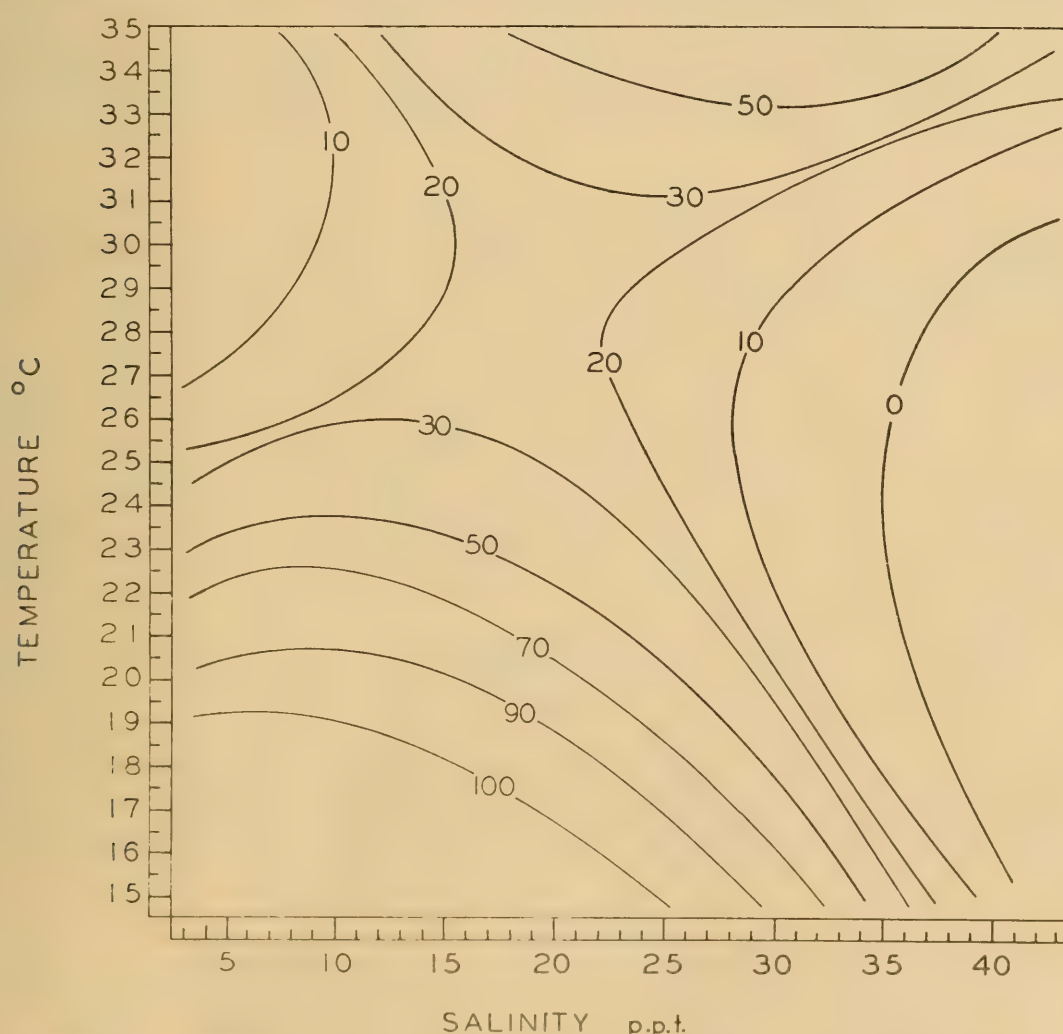


FIGURE 12. Estimation of per cent mortality of megalops stage of *Sesarma cinereum* based on the fitted response surface to observed mortality under twelve different combinations of salinity and temperature.

with no interaction gave approximately concentric circles for contours. Stage III shows a distortion of contours into pronounced ellipses. Stages IV and V show a "ridge" pattern manifesting the interaction and the lack of a unique maximum or minimum.

It is also possible to argue that the basic equations may be simplified by eliminating those factors which showed no effect and re-fitting the simpler model to the observed data. In some cases this may be desirable or even the objective of the analysis. In this study, however, an attempt was made to describe the totality of the response surface and to infer from the changing patterns with each zoeal stage a possible explanation of the mortality associated with each molt. We have, therefore, a sort of predicted mortality pattern as a function of temperature and salinity from which to postulate about the basic mechanisms involved in the system. Some such postulations which appear reasonable follow.

At 26–34 p.p.t. and 22–30° C., mortality of 10 per cent or less may be expected in the first zoeae. With lower or higher temperatures and salinities, the mortality increases as shown in Figure 8.

In the second zoeal stage the picture is quite different (Fig. 9). It was found that less than 10 per cent mortality could be expected at all temperature-salinity combinations other than 10–15 p.p.t., and 24–26° C. Even with these limits no more than 20 per cent mortality would be expected. Thus, it appears that the second larval stage is tolerant to almost any temperature-salinity condition which might exist in an estuary.

As shown in Fig. 10, the tolerances of the third zoeal stage are somewhat similar to the pattern exhibited for the first zoeal stage. While the range of temperature is only slightly changed (21–31° C.) the salinity range (23–28 p.p.t.) is more limited for a survival of 90 per cent or more.

The pattern of survival for the fourth zoeal stage does not correspond to that shown for any other (Fig. 11). As the temperature of the water increases, up to 35° C., they can withstand reductions in salinity as low as 3 p.p.t. At lower temperatures, however, mortality increases with slight changes in salinity.

The megalops stage (Fig. 12) is tolerant to a wide range of temperatures at high salinities (30–40 p.p.t.) and, interestingly enough, at high temperatures it can withstand low salinities. As the temperatures decrease, however, the resistance to low salinity is more restricted.

The successful completion of the life history of *S. cinereum* appears to depend largely on the fourth zoeal stage. That is, survival and molting to the megalops can only occur in estuarine salinities. Those which may be carried out to the high salinities of the sea, or trapped in tide pools of high salinity, would be killed. Once the megalops stage is reached, however, it can withstand the salinity of the ocean and a wide range of temperatures, and it can also survive in lower salinities at higher temperatures.

CONCLUSIONS

From a study of 1266 zoeae of *Sesarma cinereum* (Bosc), reared in the laboratory under 12 different conditions of salinity and temperature, the following conclusions may be made:

1. Hatching of the first zoea of *S. cinereum* occurred successfully at salinities of 12.5, 20.1, 26.7, and 31.1 p.p.t. None were observed to hatch as "pre-zoea."

2. Optimum salinities exist for the development of each larval stage. At 12.5 p.p.t. there was a delay in molting while at 26.7 and 31.1 p.p.t. molting proceeded more rapidly.

3. At 12.5 and 31.1 p.p.t. none of the larvae successfully completed development to the first crab stage. Mortality of zoeae at 12.5 p.p.t. was highest at the first zoeal stage and spread throughout the remaining larval stages. Mortality was highest in the fourth zoeal stage at 31.1 p.p.t. Some zoeae completed development to the crab at 20.1 p.p.t. but the highest per cent survival (24.4) to the first crab stage occurred at 26.7 p.p.t.

4. Temperature has a more definite effect on length of larval development than on mortality. At 20° C., 35–39 days were required for complete development to the first crab. At 30° C., comparable development occurred in 20–22 days.

5. Variations in temperature and salinity did not produce “extra” stages in *S. cinereum*. Those that completed development always passed through four zoeal stages and one megalops.

6. Using statistical methods, the maximum and minimum conditions for survival, the effects of salinity, temperature, and the combined effects of salinity and temperature have been postulated for each larval stage within a wide range of salinity-temperature combinations. The statistical analysis, as well as the observed results, show that salinity is the chief physical factor which confines *S. cinereum* to an estuarine environment.

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THE COMPLETE LARVAL DEVELOPMENT OF *SESARMA CINEREUM* (BOSC) REARED IN THE LABORATORY¹

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Numerous incomplete descriptions exist of larval development of the Grapsidae but they are usually based on reconstructions from planktonic material or confined to the first zoeal stage obtained from hatching in the laboratory. Cano (1891) described the development of *Pachygrapsus marmoratus* up to the crab stage but Hyman (1924) questioned the staging of the three zoeal stages by Cano (1891) and describes 5 zoeal stages from material collected at Beaufort, N. C. Conn (1884), comparing portions of larvae of *Brachyura* and *Macrura*, includes some appendages and the telson of *Sesarma* before and after hatching. Hyman (1924) gave a description of the first zoeae and appendages of *Sesarma reticulata* and, in describing the first stage of *S. cinereum*, noted that the two species are very similar morphologically. Aikawa (1929) described the first zoeae of *Sesarma* sp. briefly and figured the first stage of *S. picta* (1937), comparing setation of some appendages with the description given by Hyman. Thus only the first zoeal stage of any species of *Sesarma* has been described.

The purpose of the present study has been to rear the larvae of *Sesarma cinereum* (Bosc) in the laboratory, from hatching to the first crab stage, and provide a description of all larval stages.

The authors wish to express their appreciation to Mrs. W. A. Chipman and Mrs. Doris H. King for their assistance throughout the study. We also wish to thank Dr. Fenner A. Chace for identification of the adult females from which the eggs were obtained. Adults from which the zoeae were obtained have been deposited with the U. S. National Museum.

METHODS

Ovigerous *Sesarma cinereum* females were maintained in finger bowls containing filtered sea water until hatching occurred. The larvae were reared in groups of 10 zoeae per bowl and fed *Artemia* nauplii and *Arbacia* eggs. They were changed to freshly filtered water in clean bowls and the bowls were examined daily for exuviae. Larvae were preserved in Bouin's fixative at known intervals during development and exuviae were kept in 70 per cent alcohol to check setation of appendages.

Drawings were made to scale with the aid of a Whipple disc mounted in the ocular of a compound microscope. The larval appendages were dissected from

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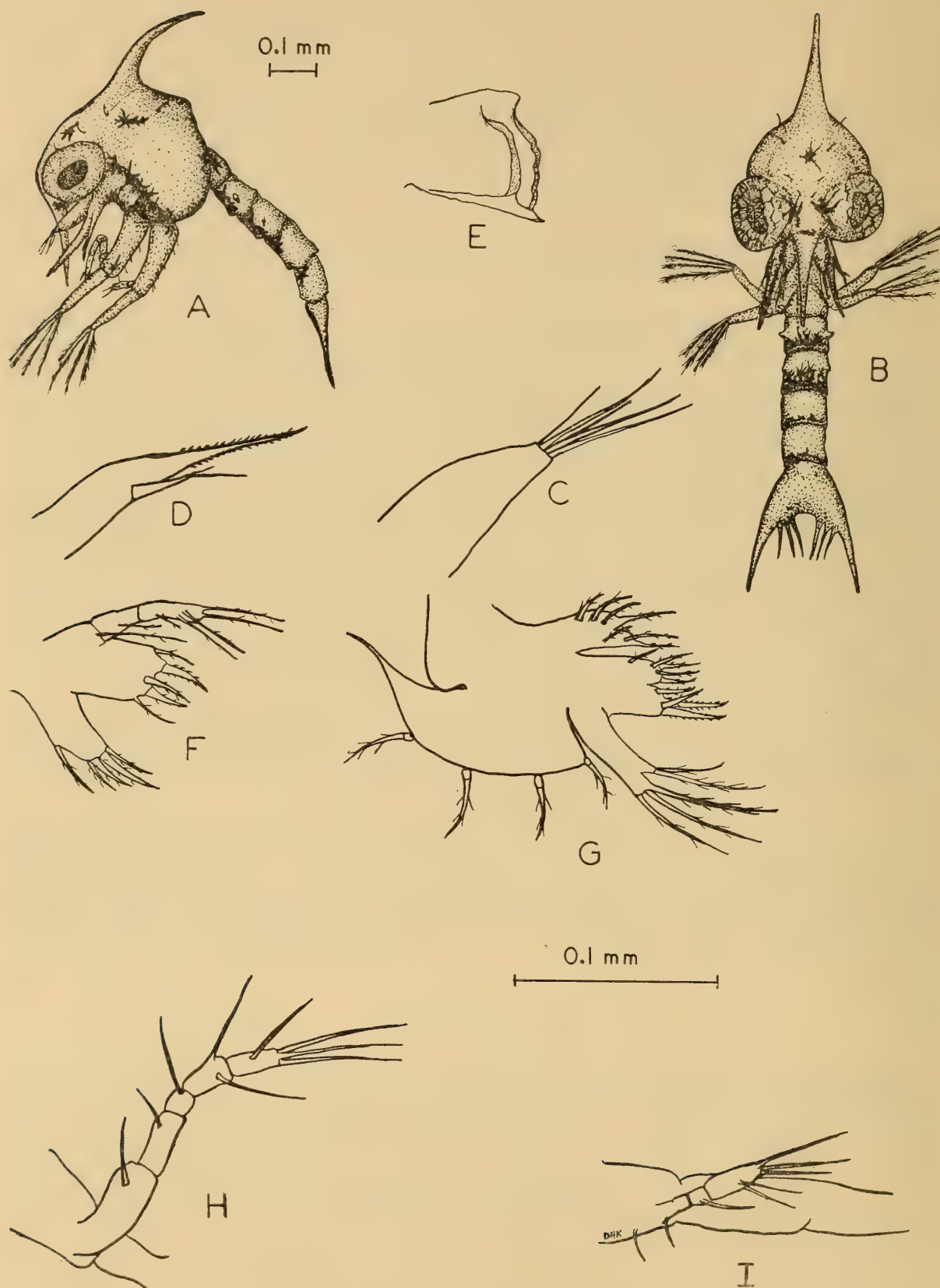


FIGURE 1. Side (A) and ventral view (B) of first zoeal stage of *Sesarma cinereum* with appendages. C, antennule; D, antenna; E, mandible; F, maxillule; G, maxilla; H, endopodite of first maxilliped; I, endopodite of second maxilliped.

each stage and drawn to a different scale from that used for the whole larvae. The chromatophore pattern was determined from living zoeae of known age.

RESULTS

There are four zoeal stages and one megalops stage in the complete larval development of *Sesarma cinereum* (Bosc). The major characteristics of each larval stage are as follows:

First zoea (Fig. 1)

The cephalothorax has a short, tapering dorsal spine which curves caudally. The rostral spine is short, approximately equal in length to the antennae and twice the length of the antennule. The eyes are not stalked. The abdomen consists of 5 segments plus the telson (Fig. 1, A). The second abdominal segment has a short lateral knob, directed laterally, and a small spine is present on the mid-lateral line of the third abdominal segment. The posterior margins of abdominal segments 2-5 terminate in short, slightly rounded lateral spines which overlap the next segment. There are 6 spines on the inner surface of the telson (Fig. 1, B). The pattern of chromatophores is consistent for all four zoeal stages of *S. cinereum*. The location of the melanophores is as follows: 1, median to the eyes, extending ventro-laterally on each side of the cephalothorax; 2, a single chromatophore, median and slightly dorsal to the eyes; 3, ventral to the heart; 4, mandibles and labrum; 5, basopodite of first maxilliped; 6, first abdominal segment, dorsal to gut; 7, postero-ventral border of remaining abdominal segments.

The short, conical antennule (Fig. 1, C) bears three terminal aesthetes of equal length and two smaller, non-plumose setae. The antenna (Fig. 1, D) consists of a protopodite which tapers gradually to a point and bears stiff hairs on the distal portion. The exopodite is a distinct segment, approximately one-third the length of the protopodite, and terminates in two, non-plumose setae of unequal length. The mandible (Fig. 1, E) has a large ventral tooth and several smaller teeth. The two-segmented endopodite of the maxillule (Fig. 1, F) bears four terminal plumose setae plus two shorter setae and a third, longer seta. The basal and coxal endites both bear 5 spines. The unsegmented endopodite of the maxilla (Fig. 1, G) is slightly bifurcated and has three terminal and two subterminal spines. The basal endite, also bifurcated, bears a total of 9 spines and 7 spines project from the coxal endite. Four soft, plumose hairs fringe the distal border of the scaphognathite and the apical tip bears one hair. The setation of the 5-segmented endopodite of the first maxilliped (Fig. 1, H) is 1, 1, 1, 2, 4, and the exopodite bears 4 plumose swimming setae (Fig. 1, B). Setation of the three segments of the endopodite of the second maxilliped (Fig. 1, I) is 0, 1, 5, and four plumose swimming setae extend from the exopodite (Fig. 1, B).

Second zoea

The eyes are stalked. A spine projects from the protopodite of the maxillule, just below the endopodite (Fig. 2, E). Seven spines project from the basal endite, and the coxal endite has 6 spines (Fig. 2, E). The endopodite of the maxilla (Fig. 2, F) bears three terminal and two subterminal setae. This arrangement

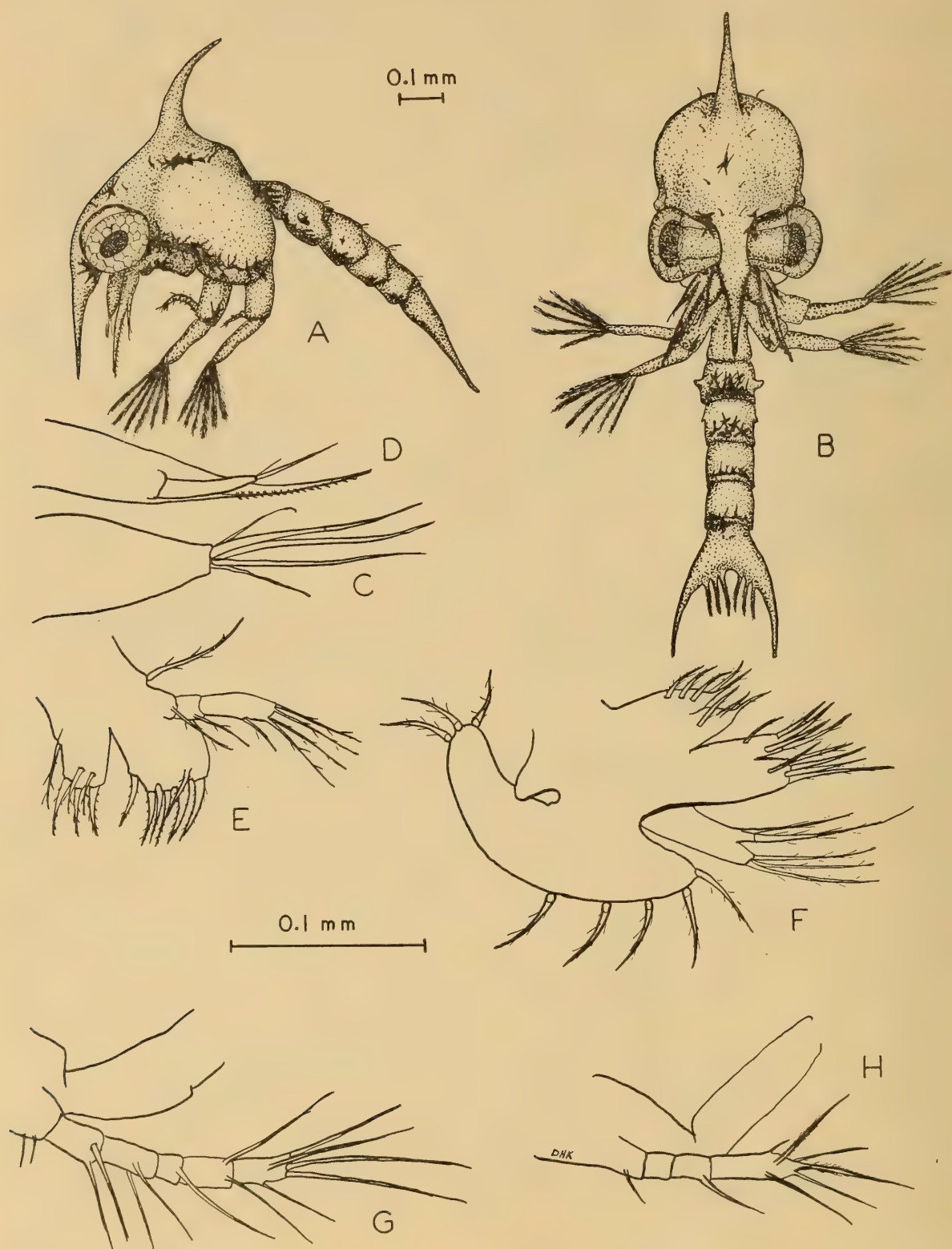


FIGURE 2. Side (A) and ventral view (B) of second zoeal stage of *Sesarma cinereum* with appendages. C, antennule; D, antenna; E, maxillule; F, maxilla; G, endopodite of first maxilliped; H, endopodite of second maxilliped.

remains unchanged in later larval stages. The endites of the protopodite are bifurcated and a total of 10 spines project from the basal endite while the coxal endite bears 8 spines. The scaphognathite (Fig. 2, F) has 5 soft hairs on the distal margin and the apical tip bears three hairs. The setation of the endopodite of the first maxilliped is 2, 2, 1, 2, 5, and 6 swimming setae are found on the exopodite (Fig. 2, B and G). On the endopodite of the second maxilliped the setation is 0, 1, 6, and the exopodite bears 6, swimming setae (Fig. 2, B and H).

Third zoea

The third maxilliped, chela and pereiopods are visible as small buds beneath the cephalothorax (Fig. 3, A). The abdominal segments have increased to 6. Segments 2–5 bear small pleopod buds. A pair of small setae fringe the posterior margins of segments 2–6 on the dorsal surface.

The endopodite bud of the antenna (Fig. 3, D) is equal in length to the exopodite but is not segmented. The basal and coxal endites of the maxillule bear 8 and 6 spines, respectively (Fig. 3, E). Ten spines are found on the basal endite of the maxilla (Fig. 3, F) and the coxal endite has 9 spines. The number of hairs on the distal margin of the scaphognathite has increased to 8 and 4 hairs project from the apical tip. Each of the exopodites of the first and second maxillipeds now bears 8 swimming setae while setation of the endopodites remains as in the second stage (Fig. 3, G and H). The rudiment of the third maxilliped is present but is unsegmented and has no setation (Fig. 3, I).

Fourth zoea

The rudimentary thoracic appendages are further developed and project below the border of the cephalothorax (Fig. 4, A). The lateral margins of the cephalothorax are fringed with short setae. Pleopod buds are larger and on segments 2–5 the buds have short setae (Fig. 4, A and B). A pair of small spines is added on the inner margin of the telson (Fig. 4, B).

The antennule (Fig. 4, C) is inflated in the basal region and terminates in 6 flat aesthetes of varying size and one short seta. The endopodite of the antenna (Fig. 4, D) is equal in length to the protopodite, terminates in one short, non-plumose spine, and is partially segmented. The mandible (Fig. 4, E) has numerous teeth on the cutting surface and a small bare, unsegmented palp is added. Eleven stiff spines project from the basal endite of the maxillule (Fig. 4, F) and the coxal endite has a total of 7 spines. The basal endite of the maxilla (Fig. 4, G) bears 12 spines and the coxal endite has 12. The scaphognathite now has a total of 23 soft hairs on the periphery. Setation of the endopodite of the first maxilliped has increased to 2, 3, 1, 2, 6, and there are 9 swimming setae on the exopodite (Fig. 4, B and H). The exopodite of the second maxilliped has 10 swimming setae. The third maxilliped (Fig. 4, J) is partially segmented and bears a few, short setae.

Megalops

Cephalothorax without rostral or dorsal spines and provided with hairs at lateral edges (Fig. 5, A and B). Center of short rostrum depressed. Abdomen of six segments and telson which bears 8 short setae on distal margin. One small

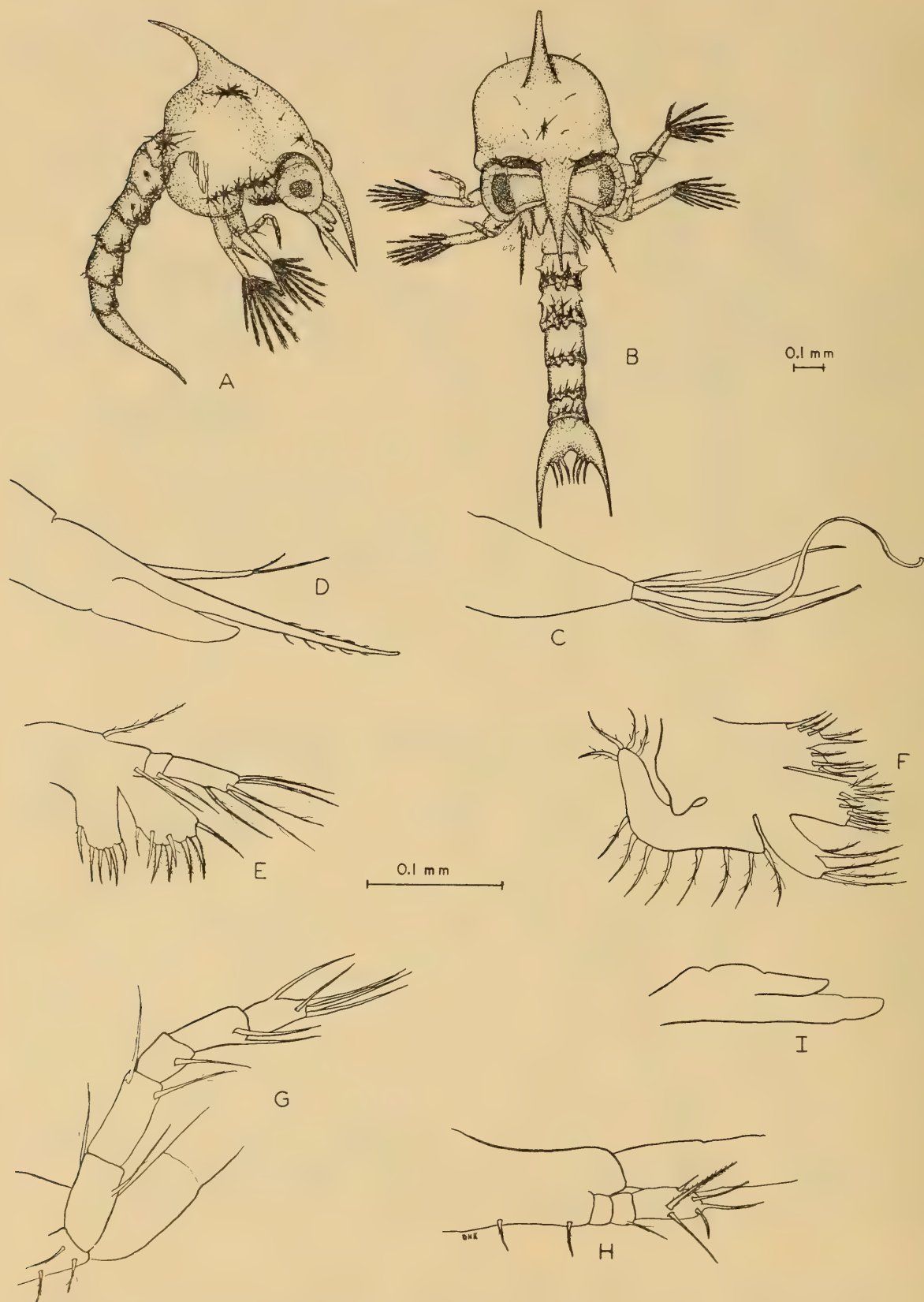


FIGURE 3. Side (A) and ventral view (B) of third zoeal stage of *Sesarma cinereum* with appendages. C, antennule; D, antenna; E, maxillule; F, maxilla, G, endopodite of first maxilliped; H, endopodite of second maxilliped; I, third maxilliped.



FIGURE 4. Side (A) and ventral view (B) of fourth zoeal stage of *Sesarma cinereum* with appendages. C, antennule; D, antenna; E, mandible; F, maxillule; G, maxilla; H, endopodite of first maxilliped; I, endopodite of second maxilliped; J, third maxilliped.

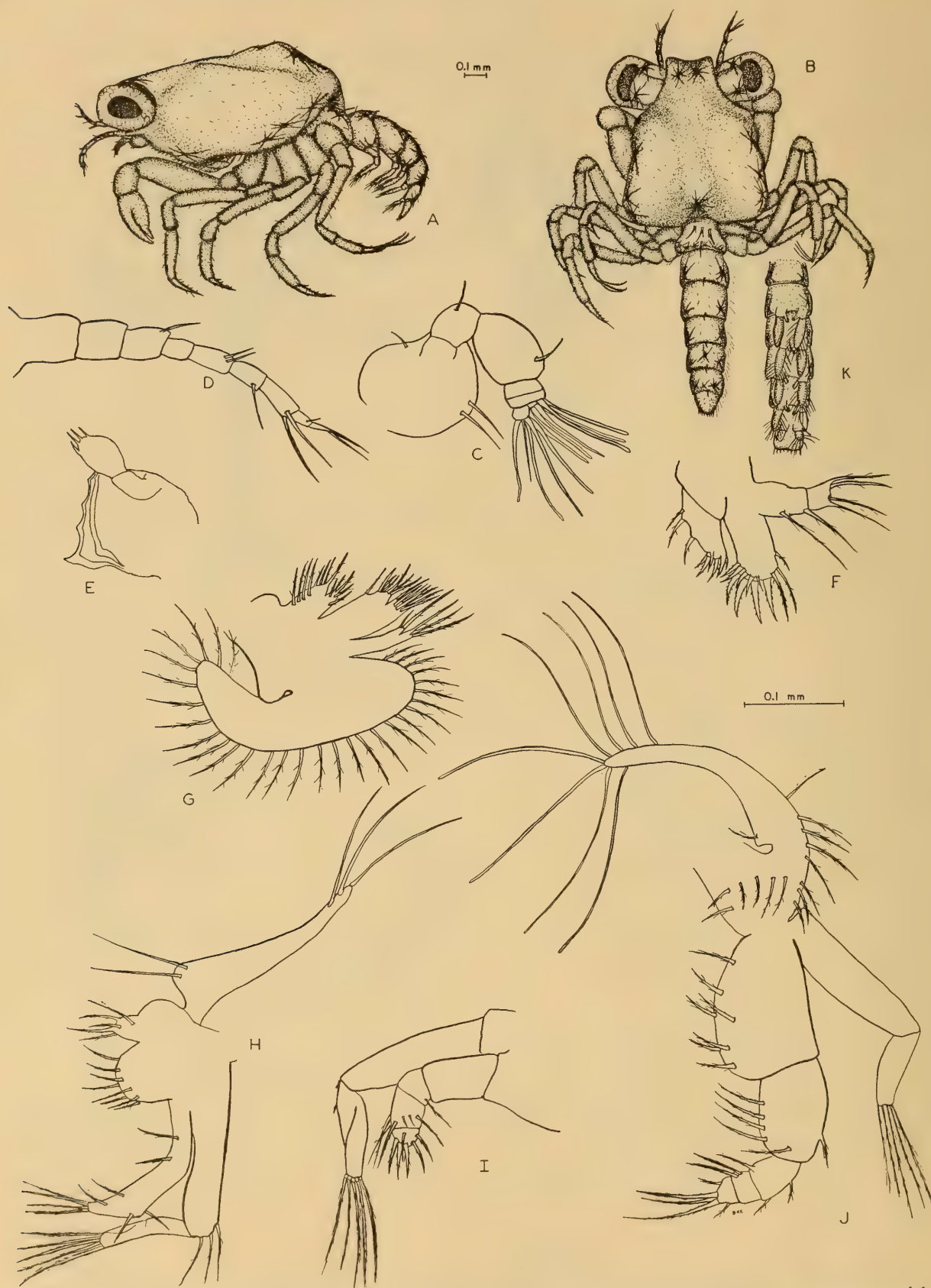


FIGURE 5. Side (A) and dorsal view (B) of megalops of *Sesarma cinereum* with appendages. C, antennule; D, antenna; E, mandible; F, maxillule; G, maxilla; H, first maxilliped; I, second maxilliped; J, third maxilliped; K, ventral view of abdominal segments.

dorsal spine on posterior margin of abdominal segments 2–6. The chromatophore pattern is as follows: 1, two on the dorsal surface of rostrum; 2, anterior and posterior peripheries of eye stalks; 3, lateral borders of cephalothorax; 4, posterior edge of carapace, dorsal to gut; 5, dorso-lateral surfaces of abdominal segments 1–6; 6, mandibles and labrum.

Antennule (Fig. 5, C) composed of enlarged base and peduncle with a flagellum of three short segments bearing a terminal tier of 5 aesthetes and a subterminal tier of 6 aesthetes. Antenna (Fig. 5, D) of 9 segments with setae concentrated on distal three segments. Mandible (Fig. 5, E) with large ventral tooth and a palp of two segments bearing four short, stiff spines on fringe of second segment. Maxillule (Fig. 5, F) with four terminal setae on endopodite and two additional setae on basal segment. Basal endite has 12 spines and coxal endite has 9 spines. Endopodite of maxilla (Fig. 5, G) with three terminal and two subterminal spines. Basal endite bears a total of 14 spines, and 13 project from coxal endite. The margin of the scaphognathite is fringed with approximately 30 soft hairs. The two-segmented exopodite of the first maxilliped has 5 terminal setae and three setae project from the first segment at the junction of the segments (Fig. 5, H). The unsegmented endopodite is reduced and bears four terminal spines. The basal endite of the protopodite has 8 prominent spines and the coxal endite bears 5. The epipodite has two setae on the basal portion and terminates in three hairs. The second maxilliped (Fig. 5, I) has an endopodite of four segments with stiff spines on the terminal segment. The two-segmented exopodite has 5 terminal setae. The third maxilliped (Fig. 5, J) has well developed endopodite of 5 segments, all bearing stiff spines. The two segmented exopodite terminates in 5 setae. The epipodite is fringed on the basal margin by numerous short spines and 8 soft non-plumose hairs arise from the distal portion.

The dactylopodite of the fifth pereopod has three long setae with minute serrations on the inner surface (Fig. 5, B). Setation of the exopodites of the pleopods of abdominal segments 2–6 is 13, 13, 13, 11, and 8. Endopodites with one short seta are found on all but the uropods. Two small hooks are located on the inner surface of all endopodites of the pleopods.

DISCUSSION

A comparison of the results of the present work with previous descriptions of *Sesarma* larvae is limited to the work of Hyman (1924) and Aikawa (1937) with first stage zoeae. Setation of the exopodites of the maxillipeds is identical for first zoea of *S. cinereum*, *S. reticulata* (Hyman, 1924), and *S. picta* (Aikawa, 1937) but, since four swimming setae are found on the maxillipeds of most first zoea described to date, the feature is of little diagnostic value. Hyman's (1924) figures indicate that some slight differences exist in setation of the maxilla. We found three terminal and two subterminal setae on the endopodite of the maxilla and Hyman (1924) figures a 3:1 setation for the first zoea of *S. reticulata*. In the present study the coxal endite of the maxilla had 7 spines and Aikawa (1937) shows 8 spines for the first zoea of *S. picta*. In previous studies on a variety of decapod larvae (Hopkins, 1943, 1944; Hart, 1935; Connolly, 1923; Costlow and Bookhout, 1959) the setation of all appendages has been shown to increase progressively through larval development. Thus, while the differences in setation of

S. cinereum, *S. reticulata*, and *S. picta* first zoea are slight, they may become greater as larval development continues.

The carapace of *S. cinereum* does not contain lateral spines. Although Hyman (1924) noted the absence of lateral spines on the carapace of first zoea of *S. reticulata* and *S. cinereum*, Aikawa (1929) groups *Sesarma* sp. with larvae of *Heterograpsus* and *Hemigrapsus* because the first zoeal stage had rostral, dorsal, and lateral spines. A later description of the first zoea of *Sesarma picta* (Aikawa, 1937) notes the absence of lateral spines. In most of these descriptions the first zoea had been figured from larvae hatched in the laboratory. The presence of lateral spines on the carapace of *Sesarma* sp. (Aikawa, 1929) must have been the result of improper identification of the adult crab.

In the present study all larvae which completed development passed through four zoeal stages. Hart (1935) described five zoeal stages from the laboratory rearing of two other Grapsidae (*Hemigrapsus nudus* and *Hemigrapsus oregonensis*) and Hyman (1924) assigned 5 zoeal stages to *Pachygrapsus marmoratus* by reconstructing the sequence of larval development from planktonic material. Hyman (1924) notes (p. 2), "The metamorphosis of the family seems to follow the usual brachyuran formula. There are at least three zoeal stages and there are probably five." Aikawa (1929) criticizes Hyman (1924) for his generalizations concerning the general structure of the grapsid larvae. The presence of four zoeal stages in the larval development of *S. cinereum* further points out the danger in generalizing on the number of larval stages a particular species of decapod "should" have. While some other grapsid crabs have been shown to have five zoeal stages, recent studies on the larval development of a variety of Brachyura larvae reared in the laboratory (Costlow and Bookhout, 1959; unpublished results) have shown that zoeal stages may range in number from 2 to 8, depending upon the species.

In the present study only one megalops stage was observed for *S. cinereum*. Hyman (1924) figures and described two megalops stages for *Pachygrapsus marmoratus*, although he does not indicate whether he actually observed the molt from the first to the second megalops or based the description on two slightly different megalops obtained from the plankton. Although only one megalops stage has been described for most crabs, Aikawa (1937) figures two stages for *Plagusia dentipes* and found three distinct modes in megalops body length. From Aikawa's (1937) account it is difficult to determine whether or not he actually observed the molt to the second megalops stage. Lebour (1928) discusses the several megalops stages attributed to *Neopanope texana sayi* by Birge (1883) and by Hyman (1925) and concludes it is probable that only one stage exists. More recent studies on laboratory-reared larvae (Chamberlain, 1957) have shown only one megalops stage for *N. texana sayi*. Although only one megalops stage may exist under optimum conditions, it is conceivable that dietary deficiencies could produce a second megalops stage in the same manner in which "extra stages" in the larval development of other species have been associated with food (Templeman, 1936; Broad, 1957).

Studies on megalops stages of other Grapsidae (Hyman, 1924; Hart, 1935; Aikawa, 1937) have figured an antennule composed of a swollen base with the distal segment of the peduncle bearing a segmented and an unsegmented flagellum. In *S. cinereum* megalops the segmented flagellum with the sensory aesthetes is

present (Fig. 5, C) but the unsegmented flagellum is missing and a small spine remains in its place. The absence of the unsegmented flagellum on the antennule may possibly be used to differentiate *S. cinereum* from the megalops of other species which have been described previously.

Recent publications on rearing larvae in the laboratory should contribute to our knowledge of larval development and to our ability to identify planktonic forms. If complete descriptions of appendages and setation are omitted, the value is considerably reduced. Without accurate descriptions of possible diagnostic features, comparative studies on planktonic material and the positive identification of larval decapods will not be possible.

SUMMARY AND CONCLUSIONS

The larval development of *Sesarma cinereum* (Bosc) has been followed in the laboratory from hatching to the first crab stage. Twelve hundred zoeae were maintained on *Artemia* nauplii and *Arbacia* eggs under constant conditions of temperature, salinity, and light. From these studies the following conclusions may be made:

1. Four zoeal stages and one megalops stage are found in the complete larval development to the first crab. These are described and figured. No variation in the number of zoeal stages was observed.

2. The setation of all functional appendages in each of the four zoeal stages has been described and figured and may be used as possible diagnostic characteristics for larvae of *Sesarma cinereum*. Setation of the maxillule and maxilla increases progressively with larval development whereas setation of the endopodites of the maxillipeds does not change with the second and third stage zoea.

3. The setation of all appendages of the megalops has been described and figured. The absence of unsegmented flagellum on the antennule of the megalops may serve as a diagnostic character for the differentiation of *Sesarma cinereum* megalops in planktonic material.

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EFFECT OF TEMPERATURE AND SALINITY ON THE OXYGEN CONSUMPTION OF TWO INTERTIDAL CRABS¹

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Studies on the effect of temperature change in poikilotherms have demonstrated physiological adaptations which permit varying degrees of independence of environmental temperatures. Various rate functions proceed within certain temperature limits, at activity levels which show compensatory responses to temperature. Mechanisms that result in such adaptations allow for degrees of thermal homeostasis in physiological processes. Latitudinally, thermal acclimation of rate functions has been demonstrated for many marine poikilotherms (Rao, 1953; Scholander *et al.*, 1953; Dehnelt, 1955, 1956; Roberts, 1957b; and many others). Seasonal acclimation to temperature is documented (Edwards and Irving, 1943a; Clark, 1955; Roberts, 1957b), as well as intertidal vertical distribution (Segal, 1956). A general conclusion, based on intra- and interspecific comparisons, may be drawn from these data. Rate functions, such as weight-specific oxygen consumption, growth, heart beat, show that northern latitude populations, at their own natural temperature, have activity rates comparable to winter-adapted populations (at other latitudes), and low intertidal populations. Further, these three groups, when compared with their ecological opposites (southern latitude, summer-adapted, or high intertidal populations), at the same experimental temperature have generally higher rates of activity. Bullock (1955) and Prosser (1955) have documented extensively these considerations for poikilotherms.

Another environmental parameter, which has been given considerable attention by physiological ecologists is the effect of salinity on animal activity. Some of these studies have concerned mainly responses of poikilotherms to various osmotic concentrations, and determinations of gain and loss of water and ions in body fluid and urine, and accompanying weight changes (Jones, 1941; Robertson, 1949, 1953; Gross, 1954, 1955, 1957; Prosser, Green and Chow, 1955).

Other studies have concerned the effect of various osmotic conditions on metabolic activity. Flemister and Flemister (1951) working with *Ocypode albicans* determined that oxygen consumption at 26° C. was lowest in sea water (378 mM. Cl/L.), which was isotonic with crab blood, this water being considerably more hypotonic than field conditions at the time of collection (480 mM. Cl/L.). As the concentration of sea water varied from isotonicity, oxygen consumption increased, the highest rates being found in hypotonic solutions. Lofts (1956) compared respiratory rates at various salinities from tap water to 65‰, of two populations of the prawn *Palaemonetes varians*, one from a low salinity environ-

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ment (1.3‰ NaCl) the other from a high salinity one (23.5‰ NaCl). In both groups the rate of oxygen consumption decreased as the salinity increased from tap water. Minimal respiratory rate for the high salinity population was at a salinity of 26‰, a condition isotonic with the animals. Minimal rate for the low salinity population occurred at 6‰. This latter value is somewhat hypertonic to the environment, and the prawns from this area are correspondingly hypertonic. Oxygen consumption increased as the experimental salinities increased beyond those of the natural environment. Gross (1955) has shown oxygen consumption to decrease as a function of desiccation in *Pachygrapsus crassipes*. Short term desiccation (several hours) is believed not to be deleterious. In a later paper, Gross (1957) measured oxygen consumption in *Uca* at 16° C. as a function of external concentration of the medium. His results showed that oxygen consumption did not always increase with osmotic stress. These results disagree with those of Flemister and Flemister (1951) for *Ocypode*. Marshall, Nicholls and Orr (1935) found that oxygen consumption of *Calanus* measured in 50% sea water and 15° C. was about 70% of respiration recorded in normal sea water (34‰). No generalization may be given regarding the effect sea water concentration has on oxygen consumption.

More recently, the combined effects of temperature and salinity on animal activity have been investigated. Broekema (1941) used different temperature-salinity combinations to determine the combined effect of these parameters on length of life of the shrimp *Crangon crangon*. She found that the optimal salinity for two-year-old shrimp was about 33‰ at a temperature of 4° C., whereas at 20° to 22° C. the optimal salinity was 28 to 29‰. When temperature decreases, salinity must rise. This condition was noted also for younger shrimp, larvae and eggs. Isotonicity is partially dependent upon temperature; at 20° C. isotonicity occurs at about 21.5‰, at 4° C., at 23‰. Further, with respect to osmotic behaviour, as temperature drops blood concentration rises in the hypotonic portion of the salinity range, and falls in the hypertonic part. These data support the fact that *Crangon* tolerates low salinities better when the temperature is high. Smith (1955a) has suggested that summer salinities in the Baltic Sea are not limiting factors for *Nereis diversicolor*, but low spring salinities and temperatures adversely affect osmoregulatory abilities, thus presenting an ecological limitation. In another paper Smith (1955b) compared chloride regulation in several geographically separated populations of *Nereis diversicolor*. One population was collected from upper River Tamar, England, and the level of chloride regulation was determined at a series of temperatures (7°, 14° and 21° C.) and over a range of chlorinities (0.02 grams chloride/liter to 14.08 grams chloride/liter). For a given chloride content of the medium the three temperatures produced no significant differences in level of chloride regulation. Further work by Smith (1957) on chloride regulation, as affected by temperature, in populations of *Nereis lighti* from the Salinas River, California has shown that at chlorinities above 1.0 grams/liter, low temperatures (0.5° C.) did not affect chloride regulation, when adapted to low salinities prior to exposure to low temperatures. However, animals adapted to 0.5° C. initially, in a chlorinity of 2.0 grams/liter, and then transferred to fresh water, failed to show chloride regulation, but did show volume regulation. At higher adaptation temperatures (12° C.) salinity reduction did not result in lowering of the coelomic chloride level.

In the last few years Kinne (1953, 1956a, 1956b) in a series of papers has reported the physiological effect of temperature and salinity on several species of invertebrates, with particular reference to growth in hydroids. Kinne (1957) states (p. 90): "temperature can change (enlarge, narrow or shift) the salinity range, and salinity can change the temperature range of a species. The effect of a given temperature depends on the salinity and vice versa." Kinne and Rotthauwe (1952) have presented data for *Rithropanopeus harrisi* showing that as temperature decreases blood concentration rises along the entire length of the curve, except for salinities above approximately 30‰. This crab is hypertonic and normally lives in waters of very low salinity (1 to 5‰). *Rithropanopeus* has been shown to withstand low salinities better when the temperature is low. It should be pointed out that these conditions are the reverse to those reported by Broekema (1941) for *Crangon*. Kinne (1956b) observed growth and reproduction in the hydroid *Cordylophora caspia* under different temperature and salinity combinations. It was determined that these hydroids withstood high temperatures at high salinities better than at lower salinities.

The combined effect of temperature and salinity has been studied with regard to its influence on temperature tolerance. The pertinent literature has been reviewed recently (Todd and Dehnel, 1960). In this laboratory Todd and Dehnel (1960) investigated the influence of seasonal change and laboratory acclimation to various temperature-salinity combinations on heat tolerance of *Hemigrapsus oregonensis* and *H. nudus*. They found that there was a seasonal change in both species when winter and summer data were compared. Further, acclimation to a high temperature increased resistance to lethal temperatures and acclimation to low salinities decreased this resistance. For both seasons, winter and summer, a combination of high temperature and high salinity proved most favorable for resistance to lethal temperatures.

The present investigation is a study of the combined effect of temperature and salinity on respiratory metabolism in two species of intertidal crabs, *Hemigrapsus oregonensis* and *H. nudus*. The facts that both species, in this geographic locality, occupy similar ecological niches, occur in abundant numbers, and are maintained readily under laboratory conditions permit inter- as well as intraspecific comparisons. It has been demonstrated that the degree (absolute and relative) to which these crabs acclimate to a given parameter, either temperature and/or salinity, depends upon the season of the year at which they were collected and the experimental temperature-salinity combination imposed at either season. This work extended from the winter, 1955, to the summer, 1957.

MATERIAL AND METHODS

Abundance and suitability to laboratory conditions of these two eurytopic species, *Hemigrapsus oregonensis* (Dana) and *H. nudus* (Dana) permit studies of temperature and salinity acclimation. Schmitt (1921) lists distribution of *H. nudus* from Sitka, Alaska to the Gulf of California, and *H. oregonensis* from Prince William Sound, Alaska to the Gulf of California.

Collections of crabs were obtained from Spanish Bank (Latitude, 49° 17' N; longitude, 123° 07' W), Vancouver, British Columbia (Fig. 1). Specific areas on the beach were marked and animals were collected only from these regions. *H.*

oregonensis was collected at approximately the 7.0-foot tide level and *H. nudus* at the 9.0-foot tide level (based on Pacific Coast Tide and Current Tables, Canadian Hydrographic Service, Department of Mines and Technical Surveys). Sea water temperatures and samples were taken at each time of collection and salinity determinations were made on these samples. Animals were returned to the laboratory in canvas buckets containing dampened sea weed.

Habitat

The Spanish Bank area, from which populations of *H. oregonensis* and *H. nudus* are studied, borders the south shore of a relatively protected bay, which extends in an east-west direction. Extension of this beach forms a point (Point Grey), along the south side of which the Fraser River flows (Fig. 1). Spanish Bank beach is a rocky mud-sand intertidal area. The habitat for these crabs is the narrow restricted upper rocky area, ranging approximately from 3.0-foot tide level to 10.0-foot tide level. The linear distance is about 150 feet. The fauna of this area is relatively poor, major elements being *Mytilus edulis*, *Balanus glandula* and the two grapsoid crabs. These species abound in numbers. Below this, the beach extends its gradual slope into a mud-sand flat which continues for several hundred yards, before dropping to form the channel. This mud flat is exposed on low tides, and a similar paucity of fauna is due to extremely low summer salinities, a condition to be discussed later.

Latitudinal distribution of *H. oregonensis* and *H. nudus* is very similar. Ecologically, however, these two species are quite different. *Hemigrapsus nudus* is an open coast intertidal species, whereas *H. oregonensis* is an intertidal bay and estuarine species. However, in this geographic area, both species are found abundantly, occupying essentially the same habitat. Vertically, *H. oregonensis* is located lower in the intertidal, and ranges from approximately the 3.0-foot tide level (lower area of rocks) to the 8.0-foot tide level. *H. nudus* is higher intertidally, ranging from approximately 6.0-foot tide level to 10.0-foot tide level. The zone of *H. oregonensis* is defined much more clearly than that for *H. nudus*. The zones as given above suggest nearly comparable width, a condition which existed at the beginning of the work, 1955–1956. For the past two years, there has been a relative stability of the *H. oregonensis* zone, but the zone of *H. nudus* has shifted progressively to a lower position, intertidally. The zone of overlap is broadening and the lower level of *H. nudus* now is approximately at the 4.0-foot tide level. At present, due to apparent contour changes of the beach, definition of areas is much less evident. Individuals of both species characteristically are found under the same rock, particularly in the area of overlap, a condition which changes somewhat seasonally. The usual niche occupied by *H. oregonensis* is under rocks, frequently partially or completely buried in the mud, which contains considerable amounts of decaying organic matter. This species is found also in beds of *Mytilus edulis*. *H. nudus* is found under rocks, infrequently in mud, and also in *Mytilus* beds, but generally occupies a much less sedimented microhabitat.

During the winter and early spring the zone of overlap is reduced and the areas occupied by the two species are defined more clearly. This zonation appears to be correlated with breeding activity. The breeding season for *H. nudus* is from January to May, and for *H. oregonensis*, from February to June. At the beginning

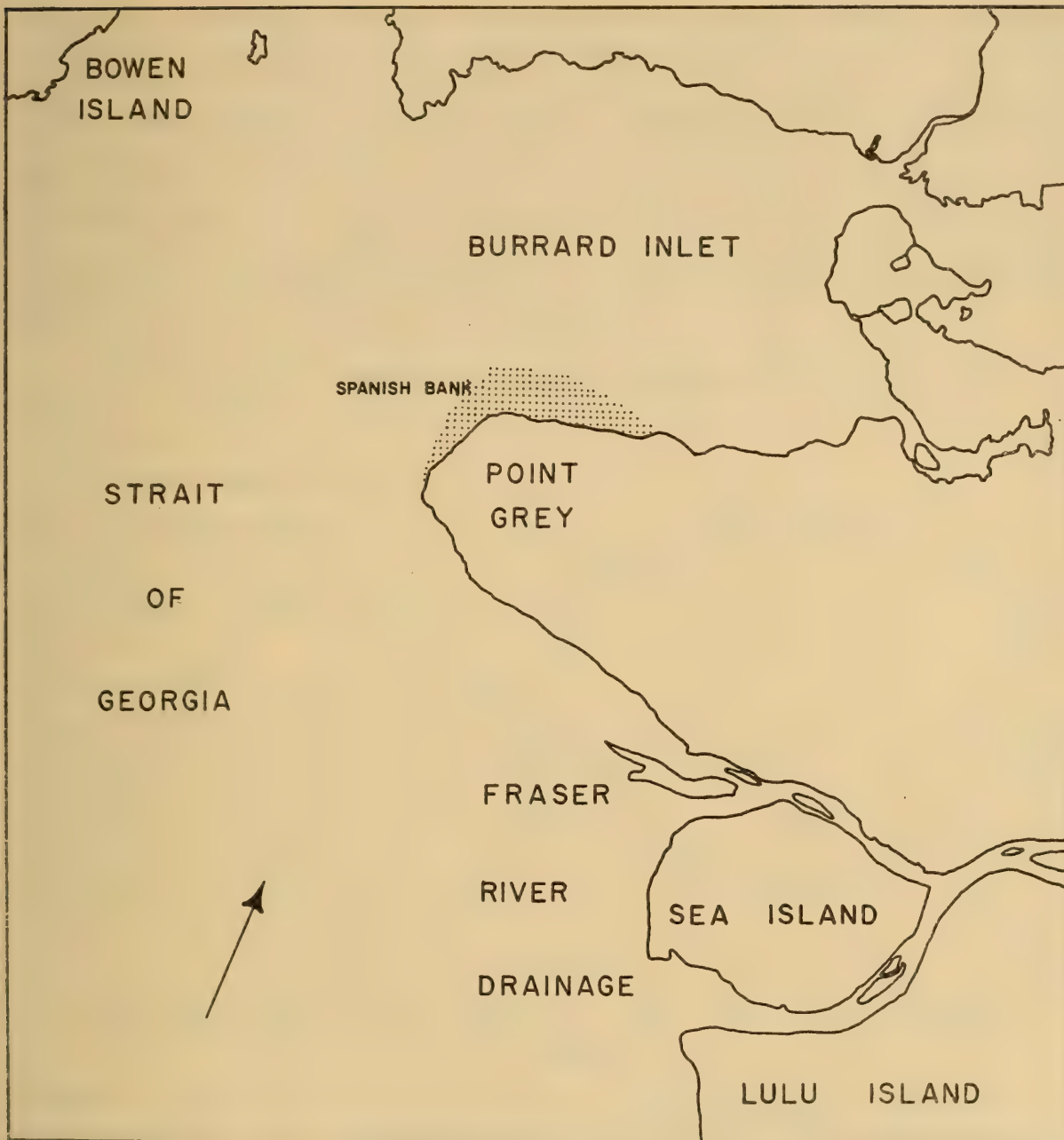


FIGURE 1. Map of the Spanish Bank area where this study was conducted. Note proximity of the Fraser River drainage which is responsible for seasonal fluctuations in salinity.

of the breeding season the two species separate spatially. After the females lay eggs, the zone of overlap is increased and the two species intermingle. Evidence is available (to be published elsewhere) which demonstrates a degree of interbreeding between these two species. An intergrade series includes crabs ranging from *H. oregonensis* with characters of *H. nudus* to the opposite extreme. In other regions of their distribution, ecological conditions such as low salinity seem to serve as barriers and prevent interbreeding. In this area, however, no such barrier apparently exists.

Temperature-salinity conditions in this area are rather severe, and warrant a

description. These relations undoubtedly serve to limit, ecologically, invasion by a more varied fauna. Seasonal variations impose strict limitations with regard to temperature and salinity tolerance, and only a select group appears to have adequate regulatory mechanisms to compensate for these changes. In adjacent regions where the Fraser River does not have an effect, the fauna is more plentiful and varied.

Previously, it was stated that this beach area extends westward and the south-facing region borders the Fraser River. Volume of this river changes seasonally, flooding in the spring and summer, due to interior British Columbia runoff. This volume of water flows into the Strait of Georgia and currents carry the low saline, low density water mass into adjacent areas and around Point Grey into the collecting

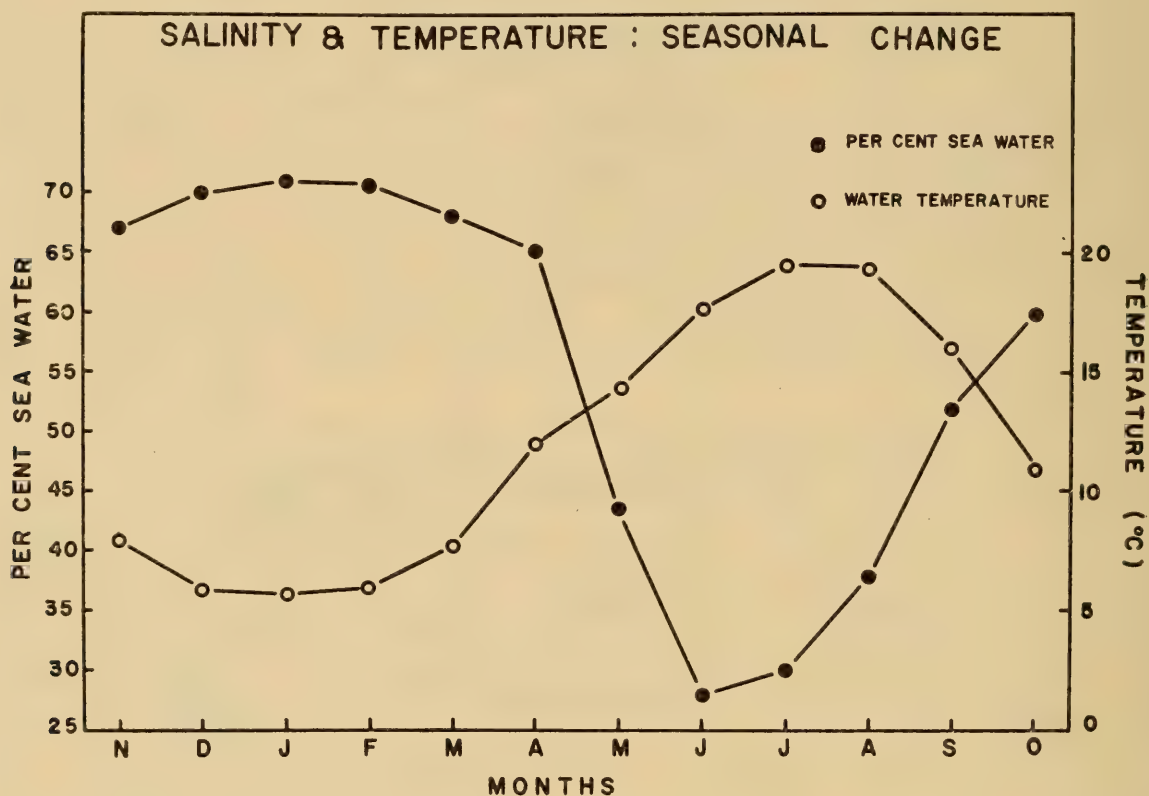


FIGURE 2. Mean monthly intertidal sea water temperatures and salinities for Spanish Bank, Vancouver, British Columbia (1955-1959). Solid circles (●) represent sea water salinity (%), open circles (○) represent sea water temperature (°C).

area. This fresh water influences greatly the intertidal salinity of Spanish Bank, not only seasonally but during a twenty-four-hour high-low tidal cycle.

During the winter, intertidal sea water temperatures range from 1.0° C. to 6° C. (Fig. 2). Isolated pools at low tide (which occur during the late evening and early morning) have been recorded as low as -0.5° C. Fraser River runoff is at a minimum and local salinities vary from 70% to 80% sea water. These conditions exist from approximately the end of November until the end of February. Winter conditions with regard to these two parameters are relatively stable ones. Spring conditions are transient, involving a rise in temperature and a drop in salinity. Temperature in late February and early March begins to rise, prior to an appreciable salinity change, from approximately 5° to 8-10° C. Following this,

salinity begins to drop to about 50% sea water. Spring conditions merge into summer conditions, which extend from approximately the end of May to the end of August. Spring temperatures continue to rise and salinities drop until average summer intertidal temperatures of approximately 20° C. and summer salinities of 25% to 35% sea water are obtained. Again, for several months relatively stable temperature-salinity conditions exist. During the fall, from approximately August to November, a transient temperature-salinity period again exists. Fall salinities rise relatively more rapidly and earlier than the seasonal lowering of temperature. Stability, low temperature, high salinity, is reached during December and the seasonal temperature-salinity cycle is completed.

In this geographical region the usual two low, two high tides per twenty-four hours are interrupted frequently and only one low or one high tide results. This condition is important conceivably during the summer months. Off shore, a line of demarcation results between low salinity, low density Fraser River water with higher salinity, higher density sea water. During a high tide, sea water over the intertidal region is mixed well, and the usual low summer salinity exists. As the tide drops, salinity remains low until the incoming tide brings in initially lower salinity water, and mixing occurs. This tidal fluctuation in salinity might be of the magnitude of 15% to 45% sea water.

Seasonal rate-temperature experiments

Male crabs of both species, ranging from approximately 0.3 grams to 6.0 grams, were collected for these experiments and were placed in plastic containers (10½" × 13" × 4½") with lids, in approximately 3.5 liters of sea water. Each container held approximately 30 animals of one species. Lids were left ajar, and a gauze cloth was placed in the container to help separate the animals. Sea water salinity approximated field salinity at the time of collection. Containers were placed in temperature-controlled refrigerators ($\pm 1.0^\circ$ C.) for approximately twenty-four hours, a sufficient time to allow partial clearing of the gut. Holding temperature approximated field conditions at the time of collection. Crabs were kept in darkness and not fed. Following the twenty-four-hour holding period, oxygen consumption was measured at a series of temperatures, 2°, (winter), 3.5°, (summer) 5°, 10°, 15°, 20°, 25°, 27°, and 30° C. In some cases oxygen consumption at two different temperatures was measured in one day. All experiments were commenced at approximately the same time each day. The following day another group was measured, these animals having been held under constant conditions of temperature and salinity for a maximum time of forty-eight hours. New collections were made to complete the series. For each experiment, measurements were recorded for twenty-four crabs. Crabs were discarded after measurements were completed for one day. Animals were changed daily with water of the appropriate salinity and temperature.

Respiration studies on individuals respiring in air can be carried out most conveniently on *H. oregonensis* and *H. nudus* because of the intertidal environment occupied. In all respiration studies measurements were made with the Wennesland modification (1951) of the Scholander microrespirometer (1949). Crabs were placed in darkened chambers with sufficient sea water to keep the gills moist, and then transferred to a constant temperature water bath ($\pm 0.1^\circ$ C.). Equilibration

time varied with the change between holding temperature and experimental water bath temperature. One hour for thermal equilibration was always allowed, with the respirometers open to the atmosphere. A further forty-five minutes to one hour was allowed for each additional 5° C. change in temperature. Following thermal equilibration, the respirometers were closed and successive readings were made at ten-minute intervals. Each experiment lasted from one and one-half hours to two hours, based on the time required for respiration levels to become linear with time. Following the experimental period crabs were removed from the respirometer chambers, dried with gauze and weighed to the nearest 0.1 gram. Data on individuals were discarded if the animals showed activity, or if high or low respiratory rates were obtained, due to approaching death. Respiration rates were given as cubic millimeters of oxygen consumed per gram per hour (weight-specific oxygen consumption). Data for each experimental temperature were plotted as weight-specific oxygen consumption as a function of weight on log-log paper. Regression lines were fitted by the method of least squares. Respiratory rate for a given weight animal was plotted against temperature on semi-log paper to demonstrate the rate-temperature relationship.

Temperature and salinity experiments

Methods of collecting and holding were similar to those described for rate-temperature experiments. For the temperature acclimation experiments, four temperatures were chosen, 5°, 10°, 15° and 20° C. ($\pm 1.0^\circ$ C.). Animals were acclimated for at least one week, maintained under dark conditions and not fed. Experiments were arranged in such a fashion that a complete series of measurements was obtained on one species prior to commencing the other. Experiments were conducted during the late spring and summer months. In one series of experiments, at four acclimation temperatures, a constant salinity of 75% sea water was used, which corresponded to winter field conditions. In another series, a constant salinity of 25% sea water was used, which corresponded to summer field conditions. At the end of one, two and in some cases three weeks, oxygen consumption measurements were made on groups of crabs acclimated to each of the four temperatures and a constant salinity. Experimental water bath temperatures used were 10° and 20° C. ($\pm 0.1^\circ$ C.). Respiratory measurements were determined as mentioned previously. After the 10° C. experimental temperature was completed, the water bath was raised to 20° C. (time required approximately one hour) and the animals were allowed one and one-half hours thermal equilibration. Following completion of the experiment and weighing, animals were returned to the experimental temperature and salinity conditions. Results were not different when animals were measured initially at 20° C. and then lowered to 10° C. Respiration rates were given as cubic millimeters of oxygen consumed per gram per hour (weight-specific oxygen consumption). Data for each acclimation temperature-salinity combination at each experimental temperature were plotted as weight-specific oxygen consumption as a function of weight on log-log paper. Regression lines were fitted by the method of least squares.

All field and experimental salinities are expressed as percentage sea water, based on a standard sea water, 31.88‰ salinity, 17.65‰ chlorinity at 25° C., as 100% sea water. Sea water concentrations were prepared from local sea water, away from the influence of Fraser River runoff, which ranged from approximately

90% sea water in the winter to 65% in the summer. For concentrations below normal sea water, dechlorinated fresh water was added until the desired dilution was obtained. For concentrations higher than normal sea water, sea salt was added, based on 31.88 grams per liter of sodium chloride. Experimental salinities were determined on a 1000-cycle conductivity bridge calibrated to the standard sea water noted above.

METHOD OF ANALYSIS

As stated previously rate of oxygen consumption was plotted as a function of body weight (weight-specific) on a double logarithmic system. Such a plot, over an adequate weight range, gives a straight line with a negative slope. Regression of weight-specific oxygen consumption against body weight assumes the form:

$$\frac{O_2}{W} = aW^{b-1}$$

or:

$$\log O_2 - \log W = \log a + b \log W - \log W$$

where O_2 is oxygen consumption of the crab in $\text{mm}^3 O_2/\text{gram}/\text{hour}$, W is body weight in grams, a the intercept and b the slope of the line. Negative linear regression coefficients were calculated by the method of least squares. It was necessary to determine whether statistically significant differences existed between regression lines representing metabolic rates of crabs measured at different combinations of acclimation temperatures (5° , 10° , 15° and 20° C.), and salinities (25% and 75% sea water) at the two experimental temperatures (10° and 20° C.). Each calculated regression line was based on oxygen consumption measurements of at least thirty-five crabs and in most cases fifty to sixty. These data are suited to statistical treatment by analysis of covariance, a standardized method outlined by Ostle (1954) for a randomized sample. The method was to test by analysis of covariance the null hypothesis that no true differences existed in the effect of different combinations of temperature and salinity on oxygen consumption, *i.e.*, that two regression lines could be represented by a single regression line. In the analysis, negative logarithm values were eliminated by multiplying oxygen consumption rates by 10 before converting to four place common logarithms.

Zeuthen (1953) has suggested that the terms "respiratory rate," "metabolic rate" and "rate of oxygen uptake" be defined as "oxygen uptake per hour per unit of body size." This procedure was observed here. Further, "weight-specific oxygen consumption" connotes the same meaning. Use of the word "acclimation" is preferred to "acclimatization" following the suggestion of Bullock (1955; see also Prosser, 1955). This word refers to intra- and interspecific compensatory changes whether these changes be phenotypic or genotypic. Other descriptive words such as "regulation," "compensation" and "homeostasis" are used with no other implications than stated above.

RESULTS

Seasonal rate-temperature experiments

Results of winter and summer oxygen consumption at various experimental temperatures, for crabs removed directly from field conditions, are shown in Figure 3 (*H. oregonensis*) and Figure 4 (*H. nudus*). Crabs of both species with a weight of 2.0 grams have been chosen arbitrarily to depict these data.

Hemigrapsus oregonensis: Respiratory metabolism for *H. oregonensis* consistently is higher for summer animals than for winter ones. These data demonstrate the fact that there is no acclimation of oxygen consumption to temperature by this species under field conditions. Shape of the winter and summer curves is similar in the physiological temperature range. Winter animals show no cold depression at 2° C. and some heat depression occurs between 25° and 27° C. Summer crabs are depressed greatly at 3.5° C., Q_{10} value between 3.5° and 5° C. is 836.0, and heat depression is above 27° C. Depression between 27° and 30° C. is somewhat less for winter crabs. Physiological temperature range for summer

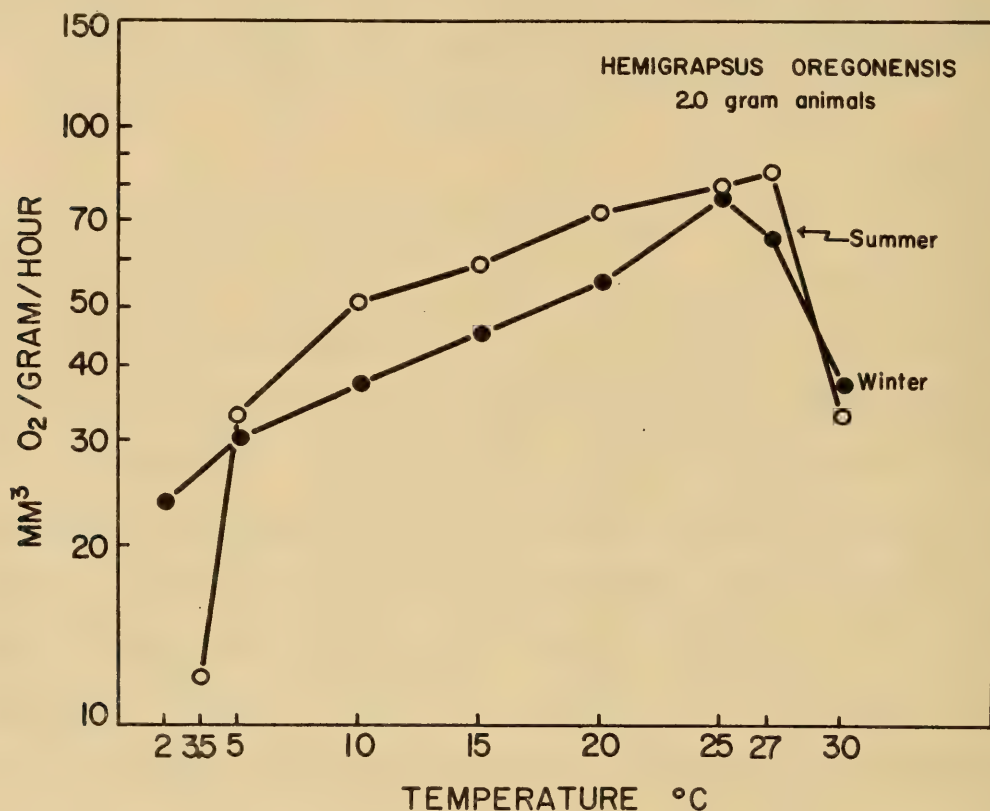


FIGURE 3. Seasonal rate-temperature curves, acutely measured, for 2.0-gram *Hemigrapsus oregonensis*. Respiratory rates for this weight crab were chosen from regression lines determined for weight-specific oxygen consumption data at each temperature. Lines were fitted by the method of least squares. Summer animals (○) were kept in 25% sea water and winter animals (●) in 75% sea water, prior to and during the experimental period.

crabs is about 5° C. to 27° C., whereas that range for winter crabs is 2° C. to 25°–27° C.

Hemigrapsus nudus: In Figure 4 approximately the same conditions as noted for *H. oregonensis* are presented for *H. nudus*. Summer-adapted *H. nudus* consistently are higher than winter ones, showing no respiratory acclimation to temperature under field conditions. Shape of the two curves is somewhat similar from 10° C. to 25° C. Consideration of the ends of the curves allows further discussion. Winter-adapted *H. nudus* show no cold depression at 2.0° C. whereas summer-adapted *H. nudus* show considerable depression below 5° C. The Q_{10} value between 3.5° and 5° C. is 166.5. At the higher end of the temperature

range, winter-adapted *H. nudus* show some heat depression between 25° and 27° C. Summer-adapted *H. nudus* likewise are depressed between 25° and 27° C. Depression between 25° and 30° C. is considerably less for winter than for summer animals. Physiological temperature range for summer crabs is about 5° to 25° C., whereas that range for winter crabs is 2° C. to 25°–27° C.

Interspecific comparison: Seasonal comparison shows that summer *H. oregonensis* have a slightly higher absolute oxygen consumption below 15° C. for any given weight and temperature, than does summer *H. nudus* for that same weight and temperature; 20° C. and above, summer *H. nudus* have a higher absolute oxygen consumption. Approximately the same rate is found at 15° C. Winter

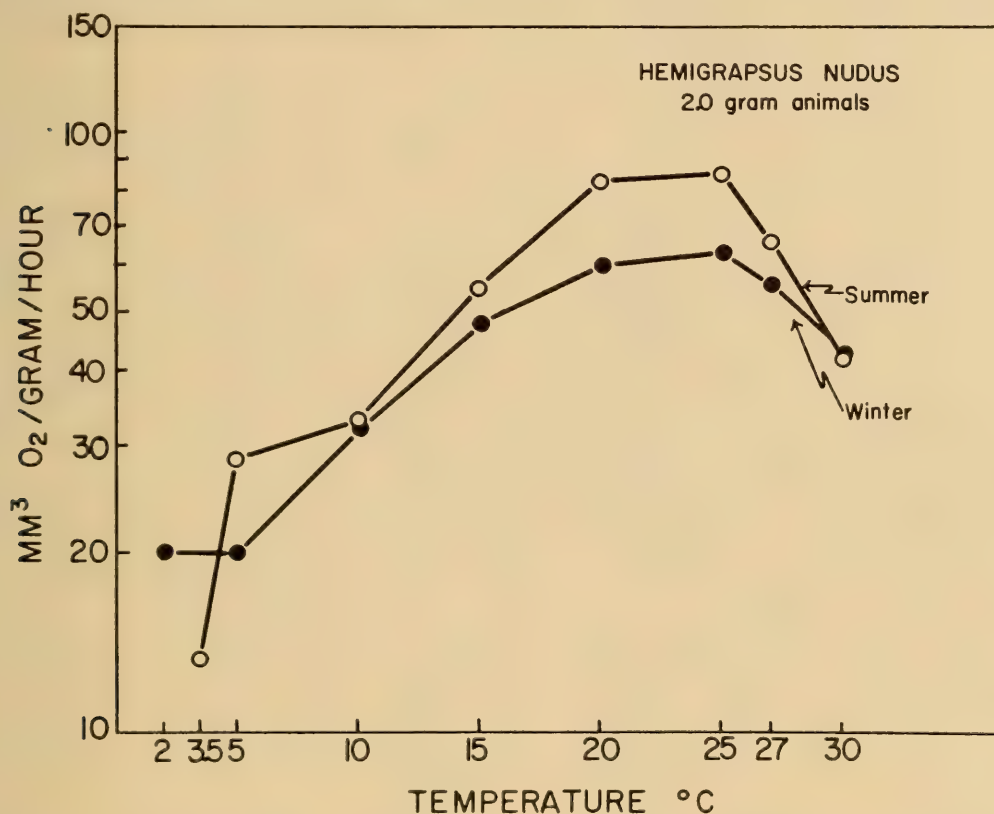


FIGURE 4. Seasonal rate-temperature curves, acutely measured for 2.0-gram *Hemi-grapsus nudus*. Respiratory rates for this weight crab were chosen from regression lines determined for weight-specific oxygen consumption data at each temperature. Lines were fitted by the method of least squares. Summer animals (○) were kept in 25% sea water and winter animals (●) in 75% sea water, prior to and during the experimental period.

crabs of both species have about the same rate of metabolism at 15° and 20° C. for any given weight. At other temperatures, the rates for *H. oregonensis* are higher.

Both of these species fail to show a shift on the ordinate, winter versus summer (an acclimation shift), but neither do they show any appreciable difference at the high end of the temperature range. It would be expected that summer animals conceivably would show depression of oxygen consumption at a higher temperature than winter crabs. Actually, winter *H. nudus* extend their upper limits to about the same level as summer ones. Summer *H. oregonensis* has a slightly higher

limit than do winter crabs. If the summer curves for both species are considered as baselines, then it can be argued that winter animals are showing an acclimation of the upper physiological limit. They do not show the drop to a lower temperature associated with cold water living. Correspondingly, winter animals have extended their curves to the left (lower temperature before depression) and winter animals have a somewhat wider physiological temperature range of oxygen consumption (2.0° C. to 25°–27° C., both species) than summer animals (5° C. to 27° C., *H. oregonensis*, 5°–25° C., *H. nudus*). Absolute temperature range for summer *H. nudus* is 20° C. and summer *H. oregonensis* is 22° C., whereas that same range for both species of winter crabs is about 23°–25° C. Such a condition is the reverse to that found by Segal (1956) for seasonal variations in heart beat of *Acmaea limatula*, and for growth rates of latitudinally displaced populations of gastropod larvae reported by Dehnel (1955).

Another method which was used for comparison between two curves is Q_{10} , provided limitations are recognized and accepted. Over physiological ranges of temperature, Q_{10} values express variations in temperature sensitivity of oxygen consumption under different thermal conditions, if intervals between temperatures at which Q_{10} values are determined are relatively small. The Q_{10} values determined for cold- or heat-depressed regions of the rate-temperature curves, for instance, would have no biological meaning unless the depression were shown to be fully reversible or to occur under normally encountered conditions. Evidence obtained from temperature tolerance experiments (Todd and Dehnel, 1960) shows that heat depression is reversible in both species, winter and summer, with respect to 50% mortality levels at much higher temperatures than reported here.

Comparison of Q_{10} values, winter versus summer, shows little consistency. Between 10° and 20° C. summer *H. nudus* have considerably higher Q_{10} values than winter ones (summer, 10°–15° C., $Q_{10} = 2.8$; winter, $Q_{10} = 2.3$; summer, 15°–20° C., $Q_{10} = 2.3$; winter, $Q_{10} = 1.6$). At other parts of the temperature range, winter crabs have higher values. Winter *H. oregonensis* between 10° and 25° C. have higher Q_{10} values (winter, 10°–15° C., $Q_{10} = 1.5$; summer, $Q_{10} = 1.3$; winter, 15°–20° C., $Q_{10} = 1.5$; summer, $Q_{10} = 1.5$; winter, 20°–25° C., $Q_{10} = 1.9$; summer, $Q_{10} = 1.2$). At other parts of the temperature ranges summer crabs have higher values.

Scrutiny of Q_{10} values for both species, winter and summer, shows many values to be much lower than the generally accepted value for poikilotherms, 2.0 to 3.0. Lower values are seen fairly consistently over physiological ranges of temperature, particularly for *H. oregonensis*, winter and summer.

Effect of acclimation temperature

Hemigrapsus oregonensis: When the four acclimation temperatures (5°, 10°, 15° and 20° C.) are compared at either low (25% sea water) or high (75% sea water) salinity at 10° C. experimental temperature, it is seen that as acclimation temperature increases oxygen consumption decreases over most of the weight range (Figs. 5 and 6). Analysis of covariance of the total of the four regression lines at either salinity, with respect to their position on the ordinate, gives a P value of less than one per cent (Table I). In the case of *H. oregonensis* (Fig. 5) when 1.0-gram crabs are compared at 5° C. and 20° C. and a salinity of 25%

sea water, there is an 87% increase at the lower acclimation temperature. The difference between these two regression lines is statistically significant ($P = 0.01$). Comparisons of the 5° C. acclimation temperature with the two intermediate temperatures similarly show significant differences at the one per cent level of

TABLE I

Analysis of covariance of rates of oxygen consumption per gram per hour as a function of weight in Hemigrapsus oregonensis and H. nudus at 10° C. experimental temperature, at two salinities (25% and 75% sea water) and four acclimation temperatures (5°, 10°, 15° and 20° C.). Combinations of acclimation temperatures are compared at each salinity. P indicates the significance of the position of the regression lines on the ordinate. P_b indicates the significance of the change in slope of the regression lines. b is the regression coefficient. r indicates coefficient of correlation

Accl. sal. (%)	Accl. temp. (°C.) comparisons	P	P_b	Accl. temp. (°C.) b	r
<i>H. oregonensis</i>	Total	0.01	0.01	5° C. —.678	— .8641
	5 and 10	0.01	N.S.		
	5 and 15	0.01	N.S.	10 —.685	— .8205
	5 and 20	0.01	0.01		
	10 and 15	N.S.	N.S.	15 —.666	— .8322
	10 and 20	0.05	0.01		
	15 and 20	N.S.	0.01	20 —.411	— .8081
	Total	0.01	N.S.	5° C. —.589	— .9345
	5 and 10	0.01	N.S.		
	5 and 15	0.01	0.01	10 —.591	— .8216
	5 and 20	0.01	N.S.		
	10 and 15	N.S.	0.05	15 —.361	— .8338
	10 and 20	N.S.	N.S.		
	15 and 20	N.S.	N.S.	20 —.333	— .5208
<i>H. nudus</i>	Total	0.01	N.S.	5° C. —.511	— .8296
	5 and 10	0.01	N.S.		
	5 and 15	0.01	N.S.	10 —.517	— .8525
	5 and 20	0.01	N.S.		
	10 and 15	N.S.	N.S.	15 —.578	— .8793
	10 and 20	0.01	N.S.		
	15 and 20	0.01	N.S.	20 —.520	— .9109
	Total	0.01	0.05	5° C. —.500	— .7598
	5 and 10	N.S.	N.S.		
	5 and 15	0.05	N.S.	10 —.441	— .8518
	5 and 20	0.01	0.05		
	10 and 15	0.05	N.S.	15 —.459	— .7022
	10 and 20	0.01	0.01		
	15 and 20	0.01	0.05	20 —.651	— .9419

probability. Further, statistical comparisons of acclimation temperatures in various combinations are given in Table I.

At the lower salinity (Fig. 5) it is seen that the regression lines, with the exception of the 5° C. acclimation temperature, converge toward the higher end of the weight range, at approximately 2.0 to 3.0 grams. Analysis of the four lines shows a statistically significant difference ($P = 0.01$) in change of slope. If the

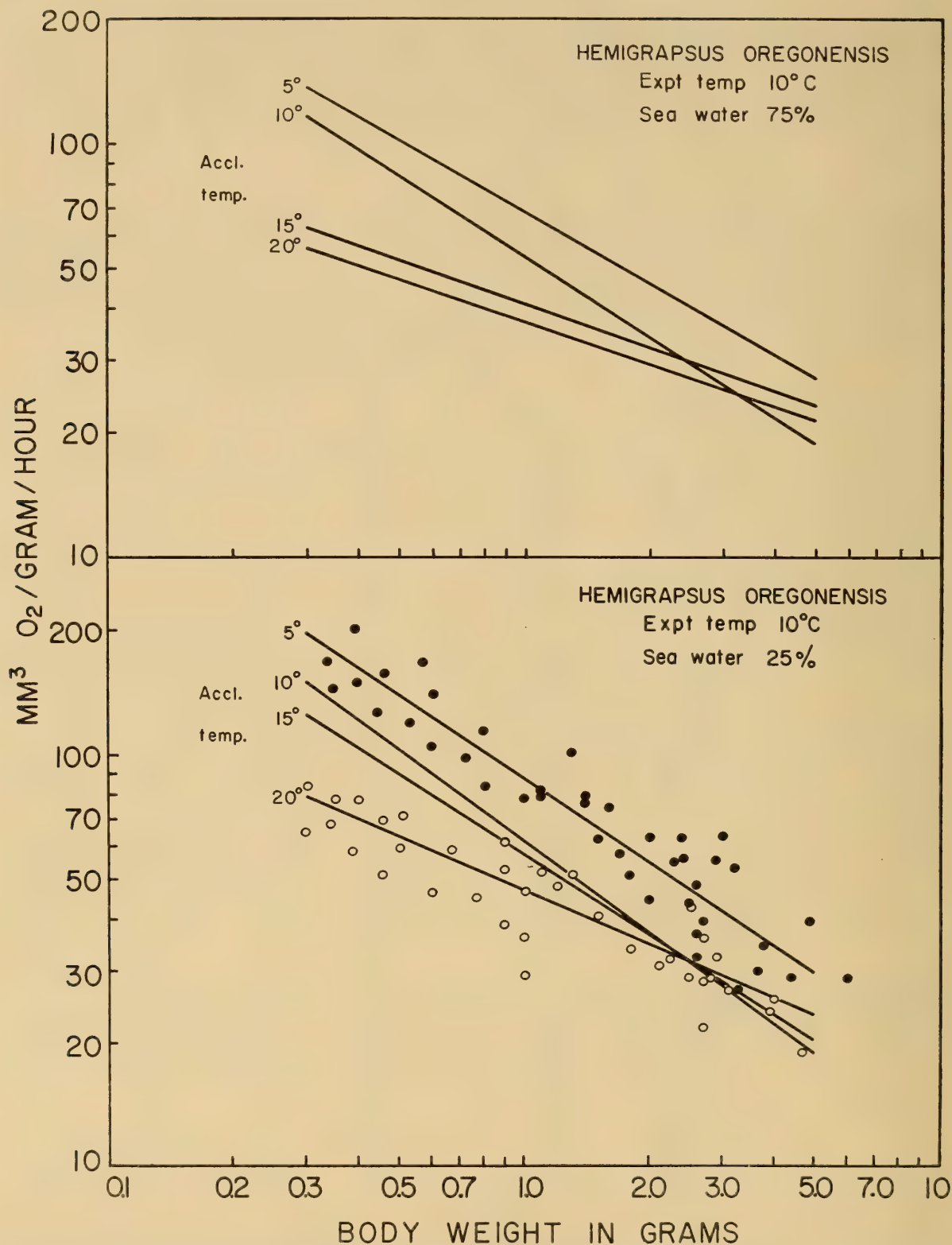


FIGURE 5. Effect of acclimation temperature on weight-specific oxygen consumption with increasing size in *Hemigrapsus oregonensis*, at the two acclimation salinities, 25% sea water (lower) and 75% sea water (upper). Each point represents an animal. Points for 5° C. (●) and 20° C. (○) are included to demonstrate variation. Regression lines were fitted by the method of least squares. Slope values, coefficients of correlation and other statistical data are given in Table I.

four acclimation temperatures are compared, as these temperatures increase the slopes of the regression lines are nearly parallel, whereas the 20° C. line is significantly different ($P = 0.01$) from the other three (Table I). If the rate of oxygen consumption of a 2.5-gram crab is compared at 5° C. and 20° C., the increase is 47% at the lower temperature. This same difference exists for the two intermediate acclimation temperatures when either is compared with the 5° C. regression line. This increase is approximately one-half that determined for a 1.0-gram animal at the low and high acclimation temperatures.

At the higher salinity, 75% sea water, *H. oregonensis* shows a similar trend to that discussed for the lower salinity (Fig. 5). Comparison of 1.0-gram animals at low and high acclimation temperatures shows an 83% increase in weight-specific oxygen consumption at the lower temperature. Further comparison of the 5° C. line with the two intermediate acclimation temperatures results in levels of probability of the same magnitude but less percentage differences (Table I). Combinations of intermediate acclimation temperatures within themselves or with high and low temperatures result in the same degrees of significance or insignificance as noted for the response to low salinity in this species.

Again as with the low salinity, slopes (b values) of the four acclimation temperatures decrease as those temperatures increase. Comparing the change of slope of the four acclimation temperature regression lines, analysis shows that there is no significant difference (Table I). Specifically, regression lines of the two lower temperatures are nearly parallel, as is the case for the two higher acclimation temperatures, and no significant differences are found to exist within these two pairs. However, comparison of only the 5° C. line with the 15° C. one gives a statistically significant difference ($P = 0.01$). There is a tendency for the 10°, 15° and 20° C. acclimation temperature lines to converge at the 2.0- to 3.0-gram weight. This is the same pattern as found at the lower salinity. Again, if a 2.5-gram crab is compared at 5° C. and 20° C. acclimation temperatures, there is found a 48% increase in oxygen consumption at the low temperature. This is approximately one-half that determined for a 1.0-gram crab.

Data are available but have not been presented for the experimental temperature, 20° C. If any of the above comparisons are made at this temperature, the same trends are observed. They differ only in the fact that regression lines for the higher experimental temperature are located at a higher position on the ordinate. At this higher experimental temperature there is no evidence of heat depression, at either experimental salinity.

Hemigrapsus nudus: Comparison of the four acclimation temperatures at low salinity for *H. nudus* demonstrates that as acclimation temperature increases, oxygen consumption decreases (Fig. 6). There is observed a 105% increase for 1.0-gram crabs at the low temperature (5° C.) when this is compared with the high one (20° C.). The only combination of two lines which is not statistically significant is the comparison of the 10° and 15° C. acclimation temperature regression lines (Table I). All other combinations give a P value of less than one per cent.

When the slopes of the four acclimation regression lines are compared it is seen that there are no statistically significant differences (Table I). The four lines assume nearly parallel relations.

At a salinity of 75% sea water response of these crabs at the four acclimation temperatures is similar when compared with the lower salinity. As the acclima-

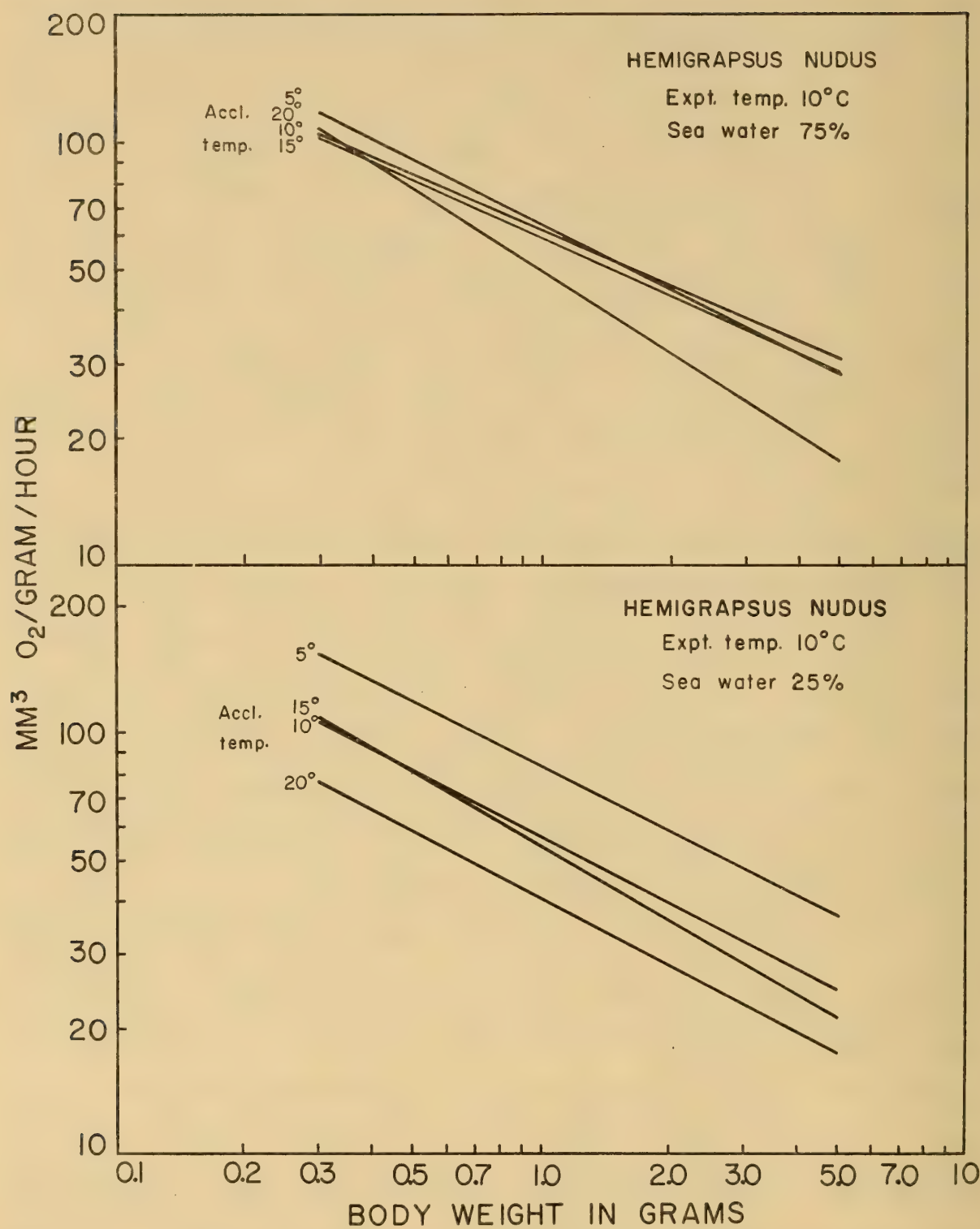


FIGURE 6. Effect of acclimation temperature on weight-specific oxygen consumption with increasing size in *Hemigrapsus nudus*, at the two acclimation salinities, 25% sea water (lower) and 75% sea water (upper). Regression lines were fitted by the method of least squares. Slope values, coefficients of correlation and other statistical data are given in Table I.

tion temperature increases, oxygen consumption again decreases over most of the weight range. Analysis of covariance of the total of the four lines shows a significant difference ($P = 0.01$). If a 1.0-gram crab is compared at high and low acclimation temperatures there is a 28% increase in oxygen consumption at the lower temperature. Further comparison of the 20° C. regression line with either the 10° C. or 15° C. line gives the same level of significance (Table I). If the 5°, 10° or 15° C. regression lines are compared within themselves, either there is no significance or a significance to the 5% level, a value considered to be statistically significant.

With regard to slope change at the higher salinity, the four acclimation temperature regression lines converge at the small end of the weight range (Fig. 6). Total line comparison for change in slope gives a significance to the 5 per cent level. This is true also for all combinations of the four regression lines with the exception of 10° and 20° C. where $P = 0.01$. There is an approximate 67% increase in weight-specific oxygen consumption for a 2.5-gram crab at 5° C. over that noted for the same weight at 20° C. This percentage increase is more than twice that observed for a 1.0-gram animal, when the same two acclimation temperatures are compared.

As in the case of *H. oregonensis* data are available but not presented for 20° C. experimental temperature for *H. nudus*. Again, the trends are the same, only the position on the ordinate differs.

Interspecific comparison: If the above results as determined by the four temperature acclimation regression lines are compared between species, at either salinity, it is observed that weight-specific oxygen consumption is similar in some instances. This is the situation at both experimental temperatures. For instance, at 25% sea water, 5° C. acclimation temperature a 1.0-gram *H. oregonensis* has an oxygen consumption per gram per hour of 87 mm.³, and the same weight *H. nudus* with identical conditions has a value of 84 mm.³ (3.5% difference). At 20° C. acclimation temperature, *H. oregonensis* has a value of 47 mm.³ and *H. nudus*, 41 mm.³ (15% difference). At 75% sea water, 5° C. acclimation temperature 1.0-gram *H. oregonensis* and *H. nudus* are about the same. At 20° C. there is a 35% difference, *H. nudus* having the higher rate (compare Figs. 5 and 6).

Effect of acclimation salinity

Hemigrapsus oregonensis: When weight-specific oxygen consumption is compared at low and high salinities at 10° C. experimental temperature and a given acclimation temperature, the higher rate is found at the low salinity (25%). Analysis of covariance of the total of the four acclimation temperature regression lines in relation to the two salinities shows a statistically significant difference ($P = 0.01$) between low and high salinity (Table II). At the 5° C. acclimation temperature (Fig. 7) a 1.0-gram crab shows a 29% increase at the low salinity over that for the same weight animal at the high salinity. Similarly, at the 20° C. acclimation temperature there is a 27% increase at the low salinity. In Figure 7 as well as Figure 8 only low and high acclimation temperatures with both salinities have been given. If either of the two intermediate acclimation temperatures

(10° and 15° C.) are compared at both salinities there is no significant difference (Table II).

There is a convergence of regression lines of each acclimation temperature at both salinities toward the higher end of the weight range. However, statistical comparison of the total acclimation temperature regression lines at the two salinities or comparison of any regression line, likewise at the two salinities, shows no significance with regard to change in slope (Table II). There is a tendency for the slope to decrease at the higher salinity for either the 5° or 20° C. acclimation temperature regression line.

TABLE II

Analysis of covariance of rates of oxygen consumption per gram per hour as a function of weight in Hemigrapsus oregonensis and H. nudus at 10° and 20° C. experimental temperatures. Each acclimation temperature is compared at the two salinities, 25‰ and 75‰ sea water. P indicates the significance of the position of the regression lines on the ordinate. P_b indicates the significance of the change in slope of the regression lines

Expt. temp. (°C.)	Accl. temp. (°C.)	P	P _b
<i>H. oregonensis</i>	Total	0.01	N.S.
	5	0.01	N.S.
	10	N.S.	N.S.
	15	0.05	N.S.
	20	0.05	N.S.
	Total	N.S.	N.S.
	5	N.S.	N.S.
	10	N.S.	N.S.
	15	N.S.	N.S.
	20	N.S.	N.S.
<i>H. nudus</i>	Total	0.01	N.S.
	5	0.01	N.S.
	10	0.01	N.S.
	15	0.01	N.S.
	20	0.01	0.05
	Total	N.S.	N.S.
	5	N.S.	N.S.
	10	N.S.	N.S.
	15	N.S.	N.S.
	20	N.S.	0.05

At the higher experimental temperature (20° C.) when any of the four acclimation temperature regression lines are compared at both salinities (5° C. at 25‰ and 75‰ sea water) there are no statistically significant differences (Table II). Similarly, there are no significant slope changes under these experimental conditions.

Hemigrapsus nudus: Consideration of the data for this species is quite different from that described for *H. oregonensis*. Weight-specific oxygen consumption measured at 10° C. experimental temperature is higher when crabs are acclimated to the low temperature, low salinity combination when compared with low temperature, high salinity (Fig. 8). Covariant analysis of the total four acclimation

temperature regression lines relative to the two salinities gives a significant difference ($P=0.01$). A 1.0-gram crab at 25‰ sea water shows a 31% increase over that same weight crab at 75‰ sea water. The lines from which this weight crab was taken are significant at the one per cent level (Table II). At the higher acclimation temperatures (Fig. 8 and Table II) crabs acclimated to the higher salinity have a higher rate of oxygen consumption and the differences at each acclimation temperature for both salinities are statistically significant ($P=0.01$). Comparison of a 1.0-gram crab acclimated to 20° C. and both salinities shows a 25% difference in rate of oxygen consumption.

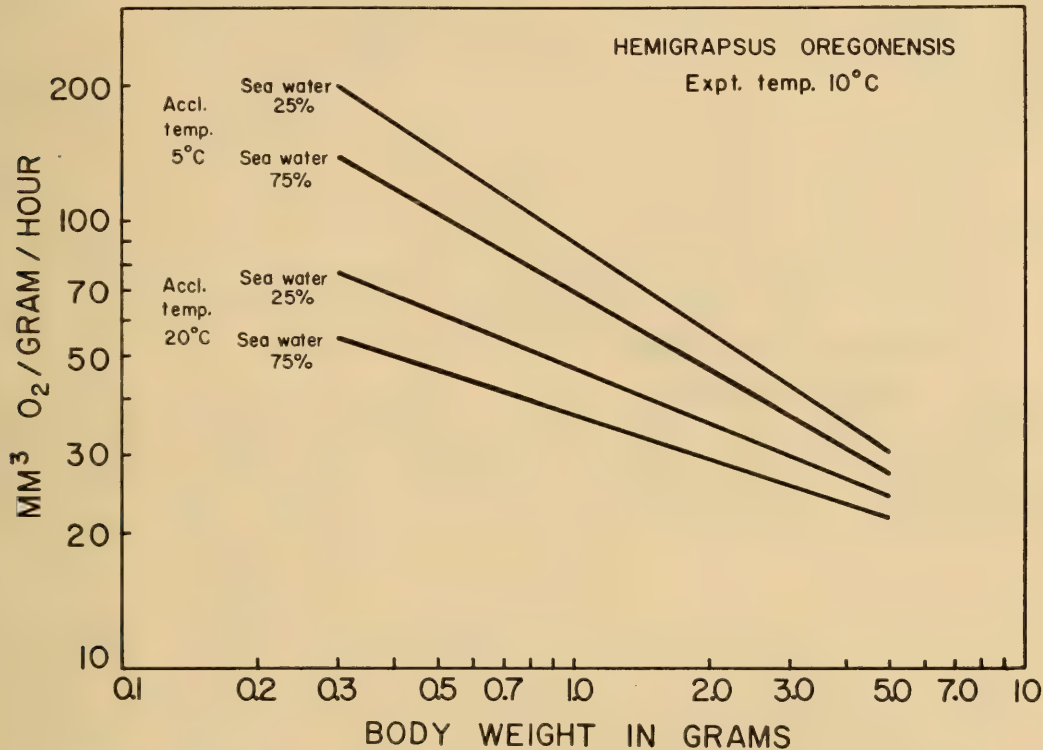


FIGURE 7. Effect of acclimation salinity on weight-specific oxygen consumption with increasing size in *Hemigrapsus oregonensis* at two acclimation temperatures (5° and 20° C.). Regression lines were fitted by the method of least squares. Slope values are given in Table I, significance of slope change and other data are given in Table II. The two intermediate acclimation temperatures (10° and 15° C.) are not included but the analyses are given in the tables.

Reference to Figure 8 and Table II indicates no significant changes in slope either for a given acclimation temperature regression line compared at two salinities or total regression lines.

The response of this species at the 20° C. experimental temperature shows no statistically significant differences when any acclimation regression line is compared at the two salinities, or when slopes are compared (Table II).

Interspecific comparison: When these two species are compared with regard to their acclimation response to low and high salinity, one basic difference is noted. When both species are acclimated to low temperature, low salinity and low temperature, high salinity the weight-specific oxygen consumption regression lines

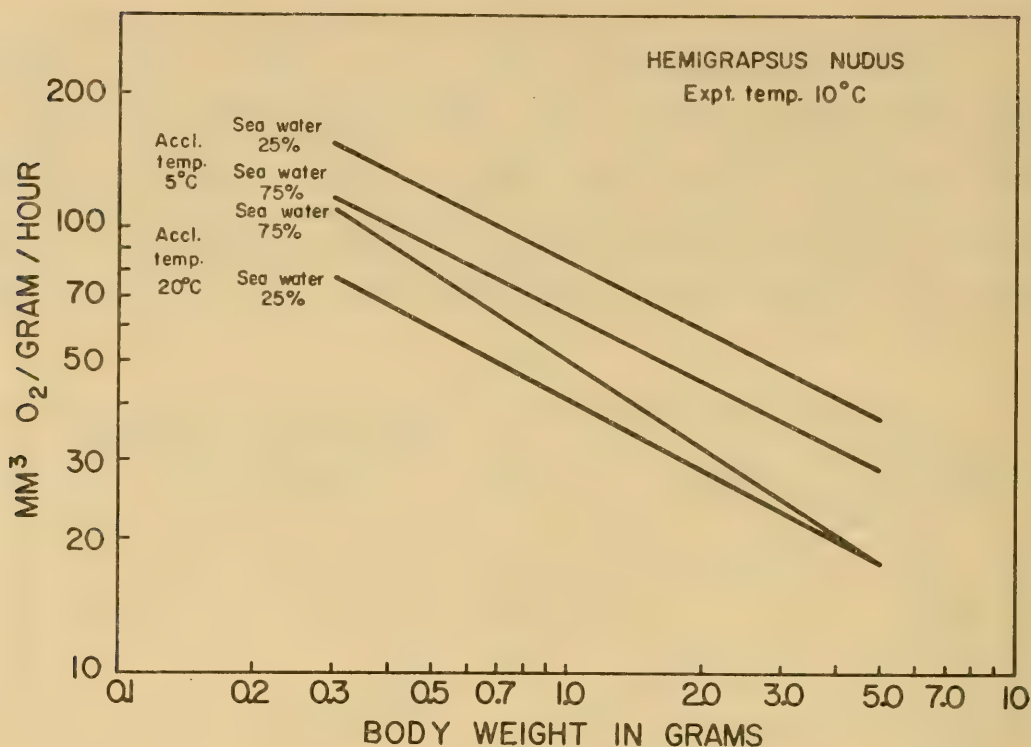


FIGURE 8. Effect of acclimation salinity on weight-specific oxygen consumption with increasing size in *Hemigrapsus nudus* at two acclimation temperatures (5° and 20° C.). Regression lines were fitted by the method of least squares. Slope values are given in Table I, significance of slope change and other data are given in Table II. The two intermediate acclimation temperatures (10° and 15° C.) are not included but the analyses are given in the tables.

are similar in position and slope. Low temperature, low salinity combination gives the greater rate. If, however, crabs are acclimated to high temperature at both low and high salinities, *H. nudus* has the higher rate at the high salinity combination and *H. oregonensis* at the low salinity combination. Percentage differences of a 1.0-gram animal for both species are approximately the same, 25%.

Effect of size

Hemigrapsus oregonensis: Reference to Figure 5 and Table I shows that there is a statistically significant change ($P = 0.01$) in slope (decrease) as the acclimation temperature increases, when crabs are measured at 10° C. experimental temperature, and maintained at the low acclimation salinity. The fact that the four regression lines converge at the higher end of the weight range shows that weight-specific oxygen consumption of small animals, except at the low temperature, is affected by increase of acclimation temperature to a greater degree than large ones. With increasing weight there is a smaller change in rate for 20° C. acclimated animals than for 5° C. ones. Thus, animals acclimated to high temperatures are less size-dependent. The Q_{10} values increase as weight increases over the range from 5° to 10° C. Comparison of a small crab (0.8-gram) with a large one (3.0-gram) shows a Q_{10} change from 1.9 to 2.4. The Q_{10} decreases with weight over temperatures from 10° to 20° C.

At the high salinity, analysis of covariance of the four acclimation temperature regression lines shows no statistical significance relative to slope change. As a result no real size effect is demonstrable (Fig. 5). The Q_{10} values at the 5° to 10° C. temperature range increase as weight increases; Q_{10} of 0.8-gram crab is 1.6, of a 3.0-gram crab, 1.9. An inverse Q_{10} relationship with weight or no change is noted at the two other temperature comparisons.

When the two acclimation salinities are compared at each acclimation temperature, relative to slope change no statistically significant differences are found (Figs. 7, 9 and Table II). Figure 9 compares different weight animals at the two salinities and over the range of acclimation temperatures. Small crabs (0.8-gram) and large ones (3.0-gram) have been chosen from the regression lines in Figure 5. If the per cent change (increase) in the rate (oxygen consumption/gram/hour) is determined for the two salinities at each acclimation temperature the following values obtain. For small crabs at 5° C. there is a 30% change in rate; 10° C., 16%; 15° C., 46% and 20° C., 23%. For large crabs (3.0-gram) the per cent change (increase) is less: 5° C., 17%; 10° C., 7%; 15° C., 4%; 20° C., 20%. It is noted that for each acclimation temperature the

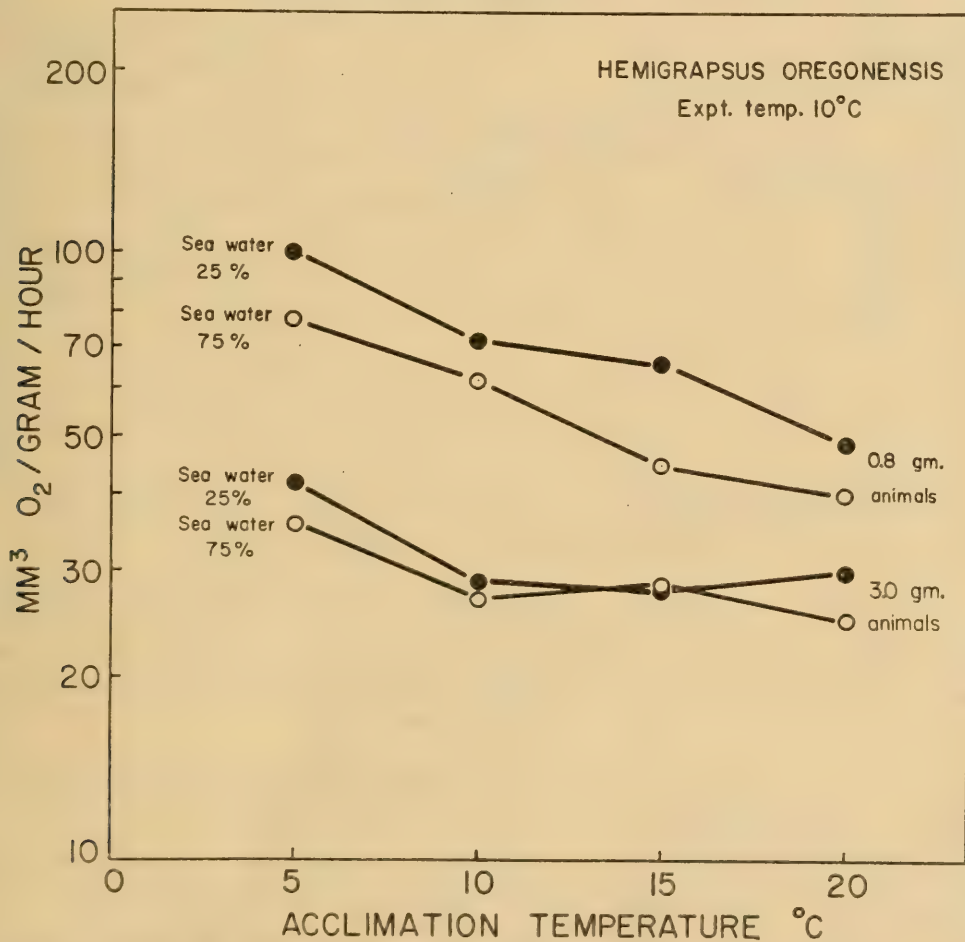


FIGURE 9. Effect of size on weight-specific oxygen consumption in *Hemigrapsus oregonensis* at the two acclimation salinities (25% and 75% sea water) and over the range of acclimation temperatures (5°, 10°, 15° and 20° C.). These weights were chosen from regression lines fitted by the method of least squares for data shown in Figure 5. The statistics for the size effect are given in Tables I and II.

rate for the low acclimation salinity is higher generally than that recorded for the high salinity.

Hemigrapsus nudus: For this species, at the low salinity, comparison of the total four acclimation temperature regression lines shows no significant slope change (Fig. 6 and Table I). Comparing Q_{10} values at the temperature interval of 5° to 10° C. shows no increase in Q_{10} as weight increases; Q_{10} of an 0.8-gram animal is 2.2; 3.0-gram crab, 2.3. Over the range from 10° to 15° C., there is only a slight

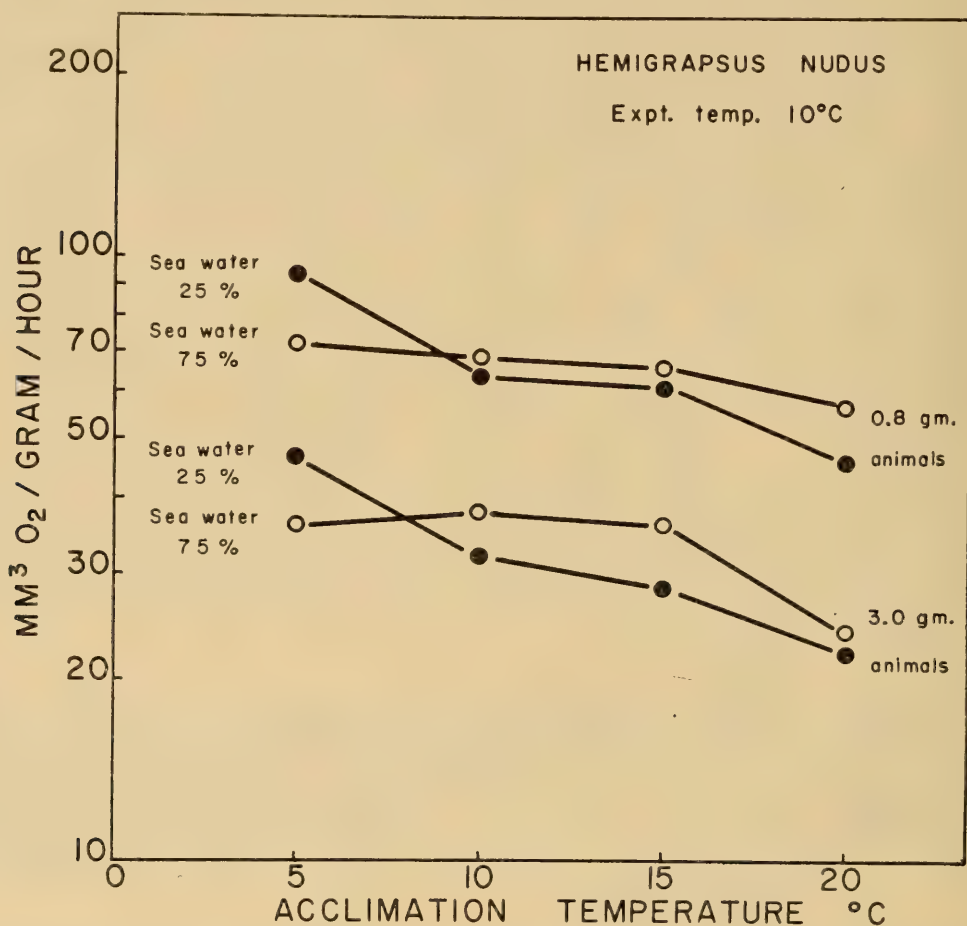


FIGURE 10. Effect of size on weight-specific oxygen consumption in *Hemigrapsus nudus* at the two acclimation salinities (25% and 75% sea water) and over the range of acclimation temperatures (5°, 10°, 15° and 20° C.). These weights were chosen from regression lines fitted by the method of least squares for data shown in Figure 6. The statistics for the size effect are given in Tables I and II.

change, 1.1 to 1.3, as there is observed over the range from 15° to 20° C., 1.8 for small crabs to 1.6 for large ones.

Covariant analysis of total four acclimation temperature regression lines at the high salinity gives a significance in slope change at the five per cent level (Fig. 6 and Table I). In this instance, however, the four regression lines converge to the left (small weights) and this demonstrates that large animals are affected to a greater extent as acclimation temperature increases. With increasing weight there is a greater change in rate for 20° C. acclimated animals than for 5° C. ones. Animals acclimated to these higher temperatures are more size-dependent. If

Q_{10} values are compared over the range from 5° to 15° C. there is no increase with weight. Over the range from 15° to 20° C., the increase is direct with size, 1.3 (0.8-gram) to 2.2 (3.0-gram).

No statistically significant differences in slope exist when weight-specific oxygen consumption for total lines is compared for both salinities at each acclimation temperature (Figs. 8, 10 and Table II). It is noted, however, that a five per cent level of significance results when rate of oxygen consumption is compared at 20° C. acclimation temperature. Again as with *H. oregonensis* small (0.8-gram) and large (3.0-gram) crabs have been chosen from the regression lines in Figure 6 and presented in Figure 10 for comparison. The per cent change (increase) in the weight-specific oxygen consumption results in the following values. For small crabs at 5° C., there is a 31% change in rate; 10° C., 10%; 15° C., 8% and 20° C., 24%. For large crabs the per cent change is, 5° C., 28%; 10° C., 19%; 15° C., 29% and 20° C., 9%.

Interspecific comparison: It can be stated generally that increase in acclimation temperature affects size differentially at low salinity in *H. oregonensis*, whereas this effect is not noted in *H. nudus*. At the higher salinity *H. nudus* shows a differential size effect, whereas *H. oregonensis* does not. When the two salinities are compared at any acclimation temperature no size effect is noted for either species. If Q_{10} values are compared, there is a general increase with weight increase in both species at the low salinity over the range 5° to 10° C. At the two higher temperature intervals generally there is a decrease in Q_{10} with size or essentially no change. At the higher salinity, over the temperature range 5° to 10° C., Q_{10} values for *H. oregonensis* increase with size, whereas there is no change for *H. nudus*. For the two higher temperature ranges Q_{10} values either decrease or remain the same for *H. oregonensis*, and increase or remain the same for *H. nudus*. For any weight crab at either salinity, Q_{10} values tend to decrease as temperature increases. An exception to this is recorded for *H. nudus* at high salinity where Q_{10} increases as the acclimation temperature increases.

Reference to Figures 9 and 10 shows that at the low acclimation temperature rate of oxygen consumption for large and small animals of both species is always higher for ones acclimated to low salinity. As acclimation temperature increases, low salinity continues to result in a higher rate for *H. oregonensis*, but for *H. nudus* higher salinity has a greater effect.

DISCUSSION

Seasonal rate-temperature experiments

Oxygen consumption measurements made on both species of crabs over a range of temperatures, summer and winter, and kept at the seasonal salinity, show that summer crabs, for any weight animal, have a higher respiratory rate than winter ones over the physiological temperature range. Further, heat depression occurs for winter animals at approximately the same high temperature as for summer crabs, and winter animals extend the range of low temperature to a point below that for summer ones. These responses result in a greater temperature range for winter animals before temperature depression occurs. The data relate to a condition described by Precht (1951) as inverse compensation (type 5). The

seasonal rate-temperature curves do not correspond to the frequently reported relationships, winter rates higher than summer (partial compensation, type 3).

Hemigrapsus oregonensis: Use of the acclimated rate-temperature curve has been suggested as a method for comparing species ecologically, and for determining whether acclimation to low temperatures shows proportionally a greater compensation when compared with high temperatures (Bullock, 1955). An acclimated rate-temperature curve can be plotted on Figure 3. This curve is determined by noting weight-specific oxygen consumption for winter animals at 5° C. (30 mm.³ O₂/gram/hour) with summer ones at 20° C. (72 mm.³). These two points represent animals seasonally adapted to their own approximate natural temperatures and measured at those temperatures. This acclimated rate-temperature curve for *H. oregonensis* has a higher Q₁₀ than either the summer or winter acutely measured curves. This means that animals show a greater temperature dependence when adapted to their field temperatures than when measured acutely over a series of temperatures, and before any demonstrable acclimation occurs. Bullock (1955) states that the acutely measured curve is steeper than the acclimated one (more temperature-sensitive). The curves for this species show this not to be the case, and if the acclimated rate-temperature curve is significant, then Q₁₀ may have no real meaning.

A further point of comparison is weight-specific oxygen consumption for summer animals at 5° C. and 30° C., and the same comparison for winter crabs at these two temperatures. Summer animals have the same rate at the low and high temperatures, and winter ones are similar. If summer and winter animals are compared together at either the low or high temperature, again their rates are nearly the same. Summer and winter animals are depressed about the same at the high temperature and no depression occurs at 5° C. These data, coupled with the acclimated rate-temperature curves, are good evidence to show that no acclimation has resulted.

Hemigrapsus nudus: When an acclimated rate-temperature curve is plotted for this species (Fig. 4) the rate for winter animals at 5° C. is 20 mm.³ O₂/gram/hour, and for summer animals at 20° C., 83 mm.³. This curve is steeper (higher Q₁₀) than the winter acutely measured rate-temperature curve, and is approximately the same as the summer curve. Thus, winter animals show a greater temperature dependence when adapted to their field temperatures. Temperature dependence for summer animals is about the same for acclimated and acutely measured rates.

When weight-specific oxygen consumption is compared for summer or winter animals at 5° and 30° C., both have a higher rate at the high temperature. But, if summer and winter crabs are compared at 30° C., both have the same rate, the depression being similar. Again this evidence shows that no seasonal acclimation has been found.

Metabolism and size

Relationship of body size to metabolism is not only recognized but is invariably the subject of controversy when this dependence is discussed in various animal groups. The general concept states that weight-specific oxygen consumption is higher for small animals when compared with large ones, measured at a given tem-

perature and determined for animals of a given species or for closely related ones. If the logarithm of the rate is plotted as a function of the logarithm of weight a linear relation exists, with a negative regression coefficient. If total oxygen consumption is plotted against body weight, large animals have a higher metabolic rate. These relationships have been shown to exist for poikilotherms and homiotherms. Absolute changes occur when intra- and interspecific comparisons are made relative to (1) temperature, (2) displacement of populations latitudinally and vertically, (3) season, (4) sex, (5) state of nutrition, (6) age, (7) other extrinsic and intrinsic factors. Relative changes remain, however, as mentioned above. Whether one is concerned with weight-specific or total oxygen consumption the problem involves the dependence of the rate on the weight, namely, the value and significance of the power function,

$$\frac{O_2}{W} = aW^{b-1} \quad \text{or} \quad O_2 = aW^b$$

The regression coefficient employed frequently is 0.67, a value that corresponds to the relationship between the surface of an animal and its body weight. This relationship indeed appears to be undoubtedly a spurious correlation as suggested by Weymouth, Crismon, Hall, Belding and Field, (1944); Brody (1945); Zeuthen (1953) and others. Regardless, it would appear ludicrous to assume the validity of such a "standard" value, particularly in view of the fact that rate functions vary with the influence of internal and external parameters. Further, there is no reason to believe that this influence on rate merely shifts the position of the regression line on the ordinate. Zeuthen (1953) has shown that the regression coefficient, b , assumes very different values ranging from 1.0 to negative numbers. Weymouth *et al.* (1944) compared respiratory rate to body weight in the kelp crab *Pugettia producta* with a series of Crustacea and found an exponent of 0.80. Scholander *et al.* (1953) likewise found a similar exponent, 0.80 to 0.85 when arctic and tropical crustaceans and fish were compared. Roberts (1957a) found for *Pachygrapsus crassipes* a power function of 0.664. Bertalanffy (1951) has correlated three different proportions of metabolism to weight with three growth types, namely $\frac{2}{3}$, $\frac{3}{4}$ and 1. Categorical definition of these power functions suggests them to be species-specific and unalterable, at least with respect to metabolic relationships. Bertalanffy and Krywienczyk (1953) demonstrated that oxygen consumption in brine shrimp, *Artemia salina*, plotted as a function of body weight follows the surface rule. These animals were cultured in artificial sea water and maintained at 25° to 27° C.

Another aspect of weight-specific respiration to body weight is the change in slope when two or more regression lines are compared, these lines resulting from measurements made under different seasonal or experimental conditions (altering one or more parameters, such as a constant temperature and two salinities). This concerns the significance of parallelism or convergence of regression lines at the low or high end of the weight range. These conditions can be discussed either by statistical analysis of b values or by comparison of Q_{10} . As regards the latter, Rao and Bullock (1954) have reviewed much of the literature. They summarize generally, stating (p. 38) that there is "a common increase of temperature coefficient with size, on the specified assumption of a weight regression." Ellenby (1951)

compared body size in the isopod *Ligia oceanica*, relative to oxygen consumption and pleopod beat. He found that total oxygen consumption at 25° C. was proportional to the 0.726 power of body weight, but that this value was not statistically different from 0.66. Oxygen consumption per unit of length² is constant over the size range (surface area is proportional to length²). Pleopod beat, on the other hand, gave a 0.66 power of body length at 15° C. and at 25° C. the value was 0.59, and these are not significantly different from the 0.5 power. In a later paper on a study of predicting oxygen consumption, Ellenby and Evans (1956) believed that in *Ligia* greater accuracy could be obtained from body weight than from a function of body length. Oxygen consumption prediction for prepupae of *Drosophila melanogaster* was found to be more accurate if based on surface area. Vernberg and Gray (1953) studied oxygen consumption of brain brei determined at 30° C. in a series of marine teleosts, and found two species whose rates of oxygen consumption were independent of weight or length.

Clark (1955) studied the effect of temperature on the oxygen consumption of the terrestrial amphipod *Talitrus sylvaticus*. He reported an exponent of 0.836 at 25° C. Further he showed that weight-specific oxygen consumption was greater in small animals (1.5 mg.) than in large ones (21.0 mg.) at temperatures above 15° C. The Q_{10} values for winter animals between 20° and 30° C. was 2.33 for small amphipods and 1.66 for large ones. Roberts (1957a) determined oxygen consumption in the shore crab *Pachygrapsus crassipes* that were acclimated to three temperatures for a period up to seventeen days. Over the weight range used (1 to 40 grams) he found that Q_{10} varied directly with weight over the acclimation temperature range 16° to 23.5° C. ($Q_{10} = 2.73$ at five grams, to 3.24 at thirty-five grams). No change was noted between 8.5° and 16° C. (Q_{10} for all weights was 2.66). At the higher acclimation temperature, slope of the negative linear regression changed from -0.336 at the two lower temperatures to -0.270 at the high temperature, a change shown to be statistically significant.

If Q_{10} values for seasonal rate-temperature data are compared for the two species of *Hemigrapsus* and for any weight of crab it is seen that generally Q_{10} values are relatively low at low temperatures in winter animals. A rise with increasing temperature to approximately 15° C. occurs, then Q_{10} values decrease as the temperature continues to rise. The same pattern exists for summer crabs except that at the lowest temperatures (3.5° to 5° C.) there is cold depression. When different weight animals are compared at any temperature interval, no generalization can be made as no trend is noted for either species, summer or winter.

Experimental results for the series of acclimation temperature and salinity combinations fail to show any definite trend of Q_{10} values with increase in size or with increase in temperature for a given size. It should be mentioned that as with seasonal rate-temperature experiments, there is a tendency for Q_{10} values to increase at lower acclimation temperatures at both salinities, and then to decrease as acclimation temperatures increase. It is well to note at this point that Rao and Bullock (1954) have shown that in many instances Q_{10} shows no trend with size. These data support this contention.

Dependence of metabolism on body size for the temperature and salinity acclimation studies is somewhat at variance with that reported in the literature. Weight-specific oxygen consumption b values for both salinities and species range

from -0.685 to -0.333 , or the positive linear regression coefficients range from 0.315 to 0.667 (Table I). Only a very few instances approached the reported 0.66 or 0.75 exponent. For the most part positive regression coefficients are relatively low values. If individual slopes or total comparison of the four acclimation temperatures at either salinity are noted, it is seen that these slopes change in some cases to a considerable degree. Statistically significant differences have been noted for *H. oregonensis* at low salinity, and *H. nudus* at high salinity. For any given acclimation temperature, scatter tends to be greatest for small crabs. As the acclimation temperature increases scatter increases along the entire weight range. Reference to Table I gives the correlation coefficients, r , of each of the acclimation temperature regression lines for both salinities and both species. In each case the value for r is significant at the one per cent level as determined from a table of significance of r (Simpson and Roe, 1939). Sample size (N) is greater than 25 for all calculated lines. It should be mentioned that regardless of increased scatter at the small end of the weight range, a straight line has been demonstrated to be statistically the best fit, as a parabolic function described less well weight-specific oxygen consumption data.

From these data it would be difficult to assign a positive linear regression coefficient to a species and suggest that such a value might be an inherent or fixed character of that species. It is evident that the regression coefficient is dependent upon past environmental histories of the animal as well as experimental variables (temperature and/or salinity) to which the animal is exposed. Variability of the response of an animal is noted to be different depending upon the weight range, temperature, particularly, and salinity combinations.

Temperature and salinity relations

In a recent paper Kinne (1956a) has discussed aspects of temperature and salinity and their biological effect on marine, brackish and fresh water animals. Generally, he believes that temperature increase intensifies activity and decreases resistance, and temperature decrease produces the opposite effect. Further, marine organisms often resist a low salinity at extremely low temperatures. The resistance and existence of marine and brackish species is facilitated in a high salinity by a relatively high temperature, and in a low salinity by a relatively lower one. Near limits of tolerance, low salinity and high temperature are often lethal, but low temperature and low salinity are tolerated. Heat resistance in brackish water species depends to a great degree on salinity. Decrease in environmental salinity causes a decrease in heat resistance, and with an increase in salinity, heat resistance increases.

Results from experiments on the effect of temperature and salinity on high temperature tolerance in the two species of *Hemigrapsus* do not support completely these ideas. It has been determined that acclimation to high temperature resulted in an appreciable increase in temperature tolerance, both in summer- and winter-adapted animals, and acclimation to low salinity decreased resistance. In this geographical area the two experimental combinations of temperature and salinity that produced the greatest (20°C. , 75% sea water) and least (5°C. , 35% sea water) resistance to high test tolerance temperatures never occur seasonally in the environment (Todd and Dehnel, 1960).

Numerous studies have demonstrated that metabolism increases when organisms are removed from their normal salinity medium and placed in a stress medium. Schlieper (1929) showed that oxygen consumption of the crab *Carcinus maenas* increased with decreasing salt concentration. Similarly, results were obtained for *Nereis diversicolor* and gill respiration of *Mytilus edulis*. Schwabe (1933) has reported a similar situation for the crayfish, *Potamobius fluviatilis*, as have Flemister and Flemister (1951) for the crab *Ocypode albicans*. These reports concern measurements made in different salinities and at a given temperature. It has been suggested that this increased rate of oxygen consumption reflected increased osmotic work. Schlieper (1929) suggested that in higher salt concentrations CO_2 , a general stimulant for cellular respiration, would be removed more readily from the animal, whereas in lower salinities there would be a tendency for CO_2 to accumulate, thus increasing respiratory rate. Later, Schlieper (1935) proposed the idea that water content of tissues was related directly to the amount of oxygen consumed. A higher water content increased volume of the tissues and the surface, which in turn facilitated absorption and hence the consumption of oxygen. This interpretation that increased metabolic rate results from increased osmotic stress has been subject to criticism. Krogh (1939) has discussed thoroughly this aspect of osmoregulation. Wikgren (1953) in an extensive review of osmoregulation as it is influenced by temperature has stated that the difference in oxygen requirement in fresh water as opposed to isotonic media was not attributable to osmotic regulation. He suggests that low salt concentrations stimulate basal metabolism directly by increased swelling of tissues or by influencing endocrine balance. These ideas in part are in accord with Schlieper (1935).

Gross (1957) reports that certain crabs, e.g., *Pachygrapsus*, show violent attempts to escape from a medium which departs much from normal sea water in concentration. He suggests that increased oxygen consumption in increased osmotic stress results from increased muscular activity. *Pachygrapsus crassipes* has been shown by Gross (1957) to be a good regulator in both hypo- and hypertonic media. This species lives in an intertidal situation in sea water of a concentration of approximately 100‰, and probably never is exposed to lower salinities in the field.

Considering the results obtained in this study, it is evident that when *H. oregonensis* and *H. nudus* are acclimated to a series of temperatures at either low or high salinity, weight-specific oxygen consumption is greatest after acclimation to the low temperature (5° C.). As that acclimation temperature increases, oxygen consumption decreases. When low and high salinities are compared at each acclimation temperature, the respiratory rate for *H. oregonensis* is higher at the low salinity, over most of the weight range for all temperatures (see Figure 7 for 5° and 20° C.). For *H. nudus* acclimated to the same conditions, the rate is higher for the low temperature, low salinity combination, but as the acclimation temperature increases, oxygen consumption is higher at the high salinity (Fig. 8). The results obtained for *H. oregonensis* might be considered to be in accord with the idea of escaping an unfavorable medium as suggested by Gross (1957), or due to increased osmotic work at lower salinities as suggested by Schlieper (1935) and others. On the other hand these ideas would not explain the results obtained for *H. nudus*. When seasonal environmental changes in this area are considered, it cannot be stated that one temperature-salinity combination is normal any more

than the reverse relations that occur at another season. This is in contradistinction to that for an open coast intertidal species. It is recalled that during the summer a low field salinity normally exists, comparable to the experimental one, and is maintained for several months. During this period the temperature is high. The animals on which these experiments were conducted were summer-adapted ones. Further, both species are exposed to the same environmental conditions in the field. In addition, animals returned to the laboratory and placed directly into low salinity sea water have never been observed to attempt to escape from that water. There has been no evidence of this at any of the acclimation temperatures with the combination of low salinity. This is also the situation for winter-adapted crabs, collected from a high field salinity and low temperature and placed either directly or gradually into low salinity water in the laboratory. Under the circumstances of summer field salinities, it seems highly improbable that the higher rate of oxygen consumption at the low salinity is the result of the attempt by these crabs to escape this low concentration of sea water, particularly since this low salinity is normal for several months of the year. *A priori*, one would expect both species to approximate the same respiratory rate, even perhaps *H. nudus* to have a somewhat higher rate. This is based on the fact that the conditions to which *H. nudus* are exposed in this locality, are the unusual ones when the ecology of this species is recalled over the latitudinal distribution. *H. nudus*, for the most part, is an intertidal species and occupies a habitat somewhat comparable to *Pachygrapsus crassipes*.

In connection with the observed high metabolic rate at low salinity, the work of Schlieper (1953, 1955, 1957) should be noted. Weight-specific oxygen consumption was determined for gills of *Mytilus edulis* collected from high salinity water (North Sea 30‰) and for ones from the same area but acclimated to low salinity (15‰) for four weeks. Respiration was higher at the low salinity by nearly a factor of 2. *Mytilus* living in low salinity water (Baltic Sea, 15‰) had a higher oxygen consumption also by a factor of approximately 2, than ones collected from the Baltic but acclimated for four weeks to high salinity water (30‰). Further, weight-specific oxygen consumption for animals acclimated to low salinity was approximately the same as that recorded for ones normally living in low salinity water, about 140 ml. O₂ per gram per hour. High salinity respiratory rate for both instances was about 80 ml. O₂ per gram per hour. This instance serves to demonstrate the fact that tissues of a *sessile* animal adapted to low salinity water have a higher respiratory rate when compared with ones adapted to high salinity water. Further, laboratory acclimation to low salinity results in a higher rate. On the other hand, measurements of mechanical activity of gill cilia, frequency of heart beat and heat resistance are lower in low salinity sea water.

Differential metabolic response of *Hemigrapsus* to salinity is believed, in part, to be the result of osmotic stress, *i.e.*, increased work to maintain an osmotic balance. At high temperatures metabolic activity increases and low salinity may be a greater stress than a high one. This low salinity could reduce resistance and cause a greater osmotic problem; the resulting high oxygen consumption reflects osmotic work. The problem may be resolved, in part, by a present study of osmotic behaviour of these two species to determine the responses to field conditions and to various laboratory combinations of temperature and salinity. Death at high temperature and low salinity may be due to osmotic breakdown. But the high temperature at which osmotic breakdown occurs depends upon the acclimation

temperature, salinity and season. Gross (1957) has calculated data from Jones (1941) to demonstrate change in osmotic gradients between blood and external medium in *H. oregonensis* and *H. nudus* as increased osmotic stresses are applied. These data show that as sea water concentration decreases below normal sea water, the internal osmotic concentration remains relatively constant.

It is of significance and interest to be able to compare different populations of the two species of *Hemigrapsus*. Gross (1955) has demonstrated the general ability of terrestrial and semi-terrestrial crabs to regulate osmotically in dilute and concentrated sea water. He has found that *H. oregonensis* and *H. nudus* from southern California can regulate in 150‰ sea water for about twenty hours. However, both species from Vancouver die at that concentration in from two to six hours. At 125‰ sea water survival is longer, one to two weeks, but mortality is relatively high during this time.

Recently Verwey (1957) has reviewed the problem of the influence of temperature on osmoregulation and has discussed means whereby marine and brackish animals attempt to live in a changing environment. For instance, the shrimp *Crangon crangon* migrates seaward toward the North Sea in autumn and returns in the spring to the region of the Dutch coast. Experimentally, it was found that salinity must increase as temperature drops for survival of this species. By migrating seaward the shrimp reach waters of higher salinity and somewhat warmer waters for winter survival, and return in the spring when coastal waters have a higher salinity. Other species that show a seasonal migration are those that migrate offshore in spring and return in autumn, such as the spider crab *Hyas araneus*, the shrimp *Crangon allmanni* and the prawn *Pandalus montagui*. This suggests that low salinities are tolerated better when the temperature is low. Still other species are those that do not migrate, the crab *Rhithropanopeus harrisi* and the amphipod *Gammarus duebeni*. These forms withstand low salinity better with a corresponding low temperature. They differ completely from *C. crangon* in their tolerance to temperature and salinity, are similar to *Hyas araneus* in tolerance, but differ from *H. araneus* in that they do not migrate. Verwey, in discussing these data, calculated changes in osmotic pressures expressed in atmospheres as opposed to the usual method of expressing salinity data as parts per thousand, freezing point depression (Δ) or per cent sea water. Expression in atmospheres relates pressure, salinity and temperature. For instance, *C. crangon* kept in a salinity of 33‰ and given a temperature change from 4° C. to 21° C., gives a value of 19.0 atmospheres for the blood at 4° C., and 19.1 atmospheres at 21° C. Sea water of 33‰ at 4° C. has an osmotic pressure of 22.99 atmospheres and at 21° C. the pressure is 24.40 atmospheres. Osmotic pressure of the blood is unchanged, sea water osmotic pressure has increased with the rise in temperature, and the gradient between blood and sea water has increased. A salinity of 33‰ is optimal for adult *Crangon* at 4° C. At a higher temperature (21° C.) the optimal salinity is about 28‰. The absolute differences expressed in atmospheres in osmotic pressure of the medium and blood at the optimal salinities for these two temperatures are the same (see Verwey, 1957, for further discussion and references). Migration of *Crangon* in the autumn results in the animal moving into water of a higher salinity and hence higher osmotic pressure, eliminating differences between blood and external medium caused by the drop in temperature, as well as the drop in salinity. It is suggested that *Crangon* attempts to maintain a

constant value in the differences between osmotic pressures of blood and medium by this migration. In connection with these aspects Panikkar (1940, 1941) found similar relationships for prawns and points out that minimum blood osmotic pressure can be lowered as temperature increases and thus osmotic work to maintain hypertonicity is less at high temperatures. He has suggested that colonization by marine animals of fresh and brackish waters in the tropics may be explained by the fact that range of tolerance to lowered salinity increases with higher temperatures. Euryhaline species can maintain hypertonicity readily and stenohaline or slightly euryhaline marine species can cope with brackish waters when temperatures are high.

The ideas suggested by Panikkar (1940, 1941) might be extended to help explain the distribution locally of *Hemigrapsus*, particularly *H. nudus*. Both species, as noted previously, breed in late winter and spring. The earliest breeding occurs during the period of low temperature and high salinity. As breeding proceeds, temperature-salinity relations change until summer conditions exist. Water currents and tides are such that zoea and megalops larvae from regions near the outer coast could be carried through the Strait of Juan de Fuca, into the Strait of Georgia. Temperature-salinity field conditions are approaching summer ones as the larvae are released by the female and become planktonic. As larvae enter the Strait of Georgia, Fraser River water is mixing with the more saline water of the Strait, and the larvae are in a low salinity environment. However, sea water temperature is rising, and with increasing temperature, tolerance to low salinity increases. Thus, perhaps larvae when exposed to low salinity, resist its effect, survive and establish themselves in an area that seasonally has low salinity and high temperature conditions. Such conditions as these might explain the invasion of *H. nudus* into this geographic area. It would appear that *H. oregonensis* occupied this area originally, and that secondarily *H. nudus* became established. Such an idea is based on the fact that the usual ecological environment of *H. nudus* is the open coast intertidal. If an east-west cline of intertidal distribution is considered, *H. nudus* is very abundant intertidally, whereas the density of the *H. oregonensis* population decreases as open coast areas are reached.

Experiments with the larvae of these two species, to determine resistance, would clarify the responses to high test tolerance temperatures, when exposed to various temperature-salinity combinations. Preliminary experiments suggest that larval responses are similar to those of the adults, and this relates favorably to environmental conditions that exist during the planktonic and settling period of *Hemigrapsus* larvae. It would be most significant to determine acclimation of oxygen consumption and temperature tolerance to different temperature-salinity combinations for populations of both species throughout their latitudinal distribution.

SUMMARY

1. Oxygen consumption measurements have been made on two species of crabs, *Hemigrapsus oregonensis* and *H. nudus*. These crabs were either brought from field conditions and measured directly, or acclimated to different combinations of temperature and salinity for two to three weeks prior to experimentation.

2. Acutely measured seasonal rate-temperature curves for summer and winter animals of both species, kept at their seasonal salinity, have shown that summer

animals at all temperatures over the physiological range have a higher weight-specific oxygen consumption than winter ones.

3. Winter animals of both species are depressed at a lower temperature than summer ones, and depression at the high temperature begins at about the same point for both summer and winter crabs. Comparison of summer and winter animals of both species at 30° C. shows about the same amount of depression.

4. Both species fail to show an acclimation shift on the ordinate, summer versus winter, but winter animals show an acclimation of the upper physiological limit.

5. Acclimated rate-temperature curves for both species generally have a higher Q_{10} than either the summer or winter acutely measured curves.

6. When summer crabs of both species are acclimated to a series of temperatures (5°, 10°, 15° and 20° C.) at either low salinity (25% sea water) or high salinity (75% sea water), it is seen that as the acclimation temperature increases, oxygen consumption decreases over most of the weight range. Statistical analysis shows these changes in ordinal position of regression lines to be significant at the one per cent level of probability.

7. In *H. oregonensis*, weight-specific oxygen consumption is higher when acclimated to 25% sea water, at all acclimation temperatures than when acclimated to 75% and at the same series of temperatures. In the case of *H. nudus* the respiratory rate is higher when crabs are acclimated to the low temperature, low salinity combination, when compared with low temperature, high salinity. At higher acclimation temperatures, crabs acclimated to 75% sea water have the higher rate. Statistical analysis of ordinal position of regression lines for both species shows them to be significant at the one per cent level.

8. When *H. oregonensis* is acclimated to low salinity and any of the four acclimation temperatures, there is a differential size effect; weight-specific oxygen consumption of small crabs shows a proportionately greater change as acclimation temperature increases. And as acclimation temperature increases, size dependence decreases. Changes in slopes (b) for the total of the four acclimation temperature regression lines are significant at the one per cent level. If crabs are acclimated to the high salinity, no size effect is demonstrable. Comparison of the two acclimation salinities at each acclimation temperature also shows no size effect. For *H. nudus*, animals acclimated to low salinity and the four acclimation temperatures show no size effect, but at 75% sea water there is a demonstrable size effect. Weight-specific oxygen consumption of large animals shows a greater rate change at higher acclimation temperatures, and there is greater size dependence at these higher temperatures. Significance of the regression lines is at the five per cent level. No statistically significant differences in slope exist when the respiratory rate is compared for both salinities at each acclimation temperature.

9. Positive linear regression coefficients for these experimental results range from 0.315 to 0.667. Only a few approach the reported 0.67 exponent. These data show that the regression coefficient is not an inherent species character, but is dependent upon intrinsic and extrinsic factors.

10. Values of Q_{10} have been used to compare seasonal rate-temperature data, but show little consistency in relation to acutely measured responses as a function of increasing experimental temperature, size increase or seasonal difference. Simi-

larly, the temperature and salinity acclimation data fail to show a definite trend for size increase or for increase in temperature at a given size. Generally, Q_{10} values increase at lower acclimation temperatures, at both salinities, and then decrease as the temperatures increase.

11. Salinity affects the metabolic response of these two species of crabs to temperature. Weight-specific oxygen consumption is highest at the low temperature, low salinity combination. As temperature increases rate of oxygen consumption remains higher at the low salinity for *H. oregonensis*, but high salinity results in a higher rate for *H. nudus*. This greater response to low salinity is thought, in part, to be the result of increased work to maintain an osmotic balance. It does not result from increased muscular activity.

12. Differential responses of *Hemigrapsus* to temperature and salinity, as measured by oxygen consumption and temperature tolerance, are suggested as a means by which *H. nudus*, in particular, became established in the geographic area of this study.

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LIMB REGENERATION AND ENDOCRINE ACTIVITY IN THE CRAYFISH^{1, 2}

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With the development of the concept of neurosecretion much new information has been obtained concerning the endocrine control of growth in crustaceans. Thus, neurosecretory cells in the x organs within the eyestalk send their secretory products via their axons to swollen axon terminations which are concentrated around a blood sinus and called the sinus gland. The secretory products are stored here for later release into the blood (Bliss, 1953; Bliss, Durand and Welsh, 1954; Carlisle, 1953; Passano, 1953). One hormone of the x organ is molt-inhibiting in crabs (Passano, 1953), and evidence has been presented which indicates that it is produced in the crayfish by the type 2 neurosecretory cells in the x organ (Durand, 1956). The specific roles of other neurosecretory cells in the brain and eyestalks, some of which send their axons to the sinus glands, are unknown. Work on crustacean neurosecretion has been reviewed recently by Knowles and Carlisle (1956).

In addition to neurosecretory elements, another endocrine gland, the y organ, is important in the regulation of crustacean growth. This gland, first described by Gabe (1953), produces a hormone which has been shown to stimulate growth and molting in *Carcinides* (Echalier, 1954, 1955).

Because both of these endocrine glands function in the control of growth, it is important to know the time relationships between their periods of activity. The secretory activity of the two organs was studied, therefore, along with the regeneration of autotomized limbs since Bliss (1956) showed that certain stages in the regeneration of limbs in *Gecarcinus* reflect growth conditions within the animal in general.

MATERIALS AND METHODS

Animals

Young *Orconectes limosa* (12–15 mm. carapace length) were collected from the Rahway River, Rahway, N. J. during the summer of 1956. Crayfish were then moved to Camden, N. J. and kept in half pint paraffin-lined containers, one crayfish per container. The containers were covered with translucent plastic and kept in a water bath (temperature 20–25° C.). The water in the containers was changed twice weekly during July and August and once a week during September

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and October. Freshly collected duckweed and algae, upon which the animals feed, were added to the containers each time the water was changed.

Regeneration

In one group of animals, either on the day of molt or on the day after molt, each crayfish was made to autotomize its right rear walking leg. Pinching the meropodite with fine forceps was usually a sufficient stimulus to bring about autotomy. Animals were examined for signs of regeneration of the limb about three times per week during July and August and twice a week thereafter to the middle of October. A regenerating limb grows out from the autotomy plane as a long thin flexible structure, its segments linearly arranged within a membranous sac. This membranous sac and its contents, hereafter referred to as a limb bud, was measured under $15\times$ magnification with a pair of dividers, and the length was taken by comparison with the half-mm. scale of a metal machinist's rule. The limb bud was measured from the tip to the plane of autotomy.

In another group, crayfish were kept in the laboratory as described above and were made to autotomize the right hind walking leg within two days of collection. A record of the regeneration of the leg was made for each crayfish as described.

In order to compare the rates of limb regeneration of different sized animals, the size of the regenerating limb is expressed as the per cent of the carapace length:

$$R = \frac{\text{length of regenerating limb}}{\text{length of carapace}} \times 100.$$

This formula is comparable to that used for studies of limb regeneration in *Gecarcinus lateralis* (Bliss, 1956).

Histology

Eyestalks and y organs were fixed in Bouin plus 1% CaCl_2 at various stages of limb regeneration and embedded in Tissuemat. Eyestalk serial sections (7μ) and y organ serial sections (10μ) were stained in most cases with the aldehyde fuchsin technique (Halmi, 1952) with modifications as suggested by Dawson (1953).

Counts of the type 2 neurosecretory cells which were located in the x organ and which contained secretory material were made according to the method given in a previous paper (Durand, 1956).

RESULTS

Limb regeneration

Studies of the regeneration of autotomized limbs were carried out in this investigation. The rate of limb regeneration was shown earlier (Bliss, 1956) to vary at certain stages of the intermolt period, and it was hoped that limb regeneration curves might be used as an indicator of endocrine conditions in the animal in general. As will be seen, it was found that only at certain times during the intermolt period did the rate of limb regeneration reflect changes in endocrine conditions in the animal.

Typical curves of limb regeneration are shown in Figure 1. These are selected curves for individual crayfish. The animals were made to autotomize on the day

after collection, and the rate of limb regeneration was measured to the following molt. Since the animals were collected at random, it is believed that all stages of the intermolt period were represented in the population at the beginning of the experiments. Therefore, Figure 1 illustrates limb regeneration which began at successive stages of the molt cycle.

It is seen that the stages of limb regeneration are similar to those reported by Bliss (1956) for *Gecarcinus*. Thus, following autotomy there is a *lag period* before any visible signs of regeneration occur; this period probably represents the time required for healing processes and the general mobilization of local growth

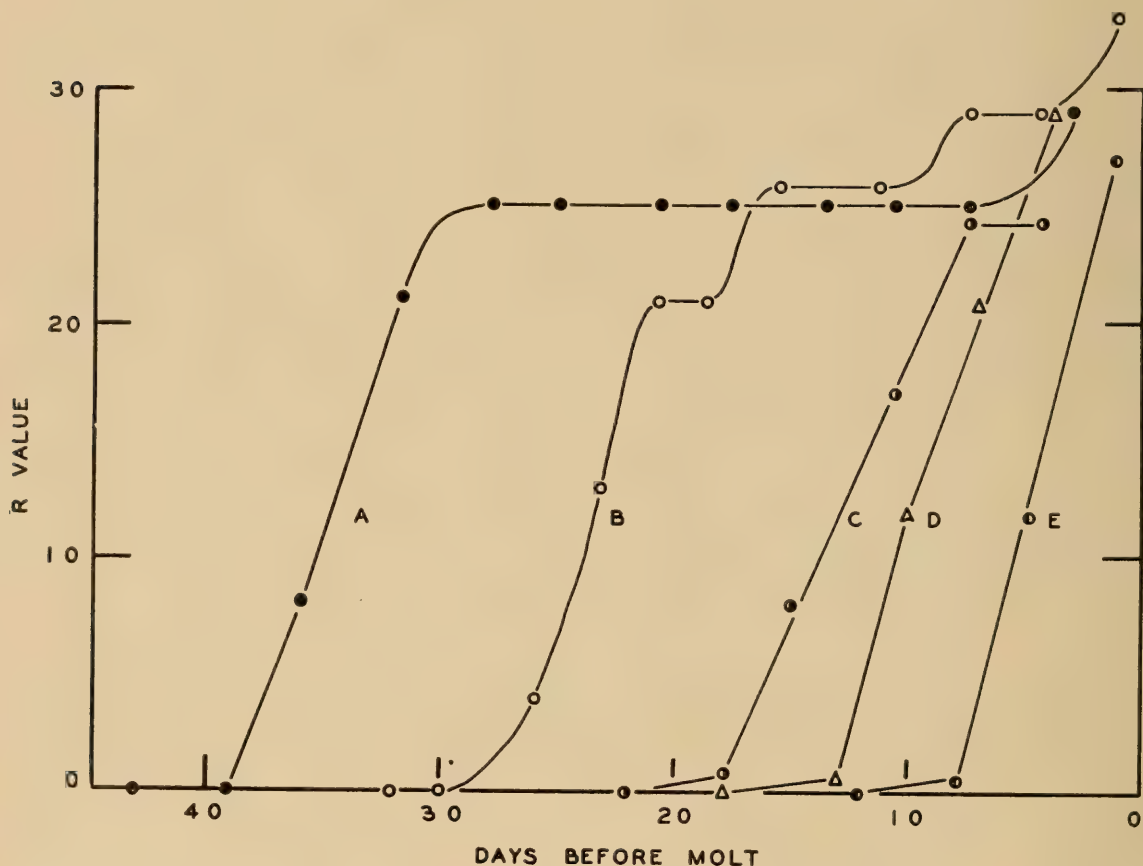


FIGURE 1. Limb regeneration curves for selected crayfish.

processes. It is interesting that the length of the lag period (mean, 6 days) is essentially constant no matter when autotomy occurs during the intermolt period.

Next, there occurs a period of *basal growth*, during which time the regeneration of the limb progresses rapidly. This stage is also essentially the same (the slopes are the same) no matter when it occurs during the intermolt period. The mean slope of the basal growth stage was 2.2, and 10–11 days were required for the completion of basal growth.

Following basal growth, linear growth of the regenerating limb ceases, that is, it enters a period of *plateau* which lasts until near the end of the intermolt period. The limb has usually reached about 22% of the carapace length at this time. According to Hodge (1956) differentiation of limb tissue continues during the period

of plateau. Therefore, only increase in length ceases while development does continue. The plateau R value is higher in *Orconectes* than it is in *Gecarcinus* (crayfish 22, crab 10). Bliss (personal communication) has suggested that this might be a reflection of the fact that the segments of the crayfish limb bud are straight whereas those of the crab are folded upon one another.

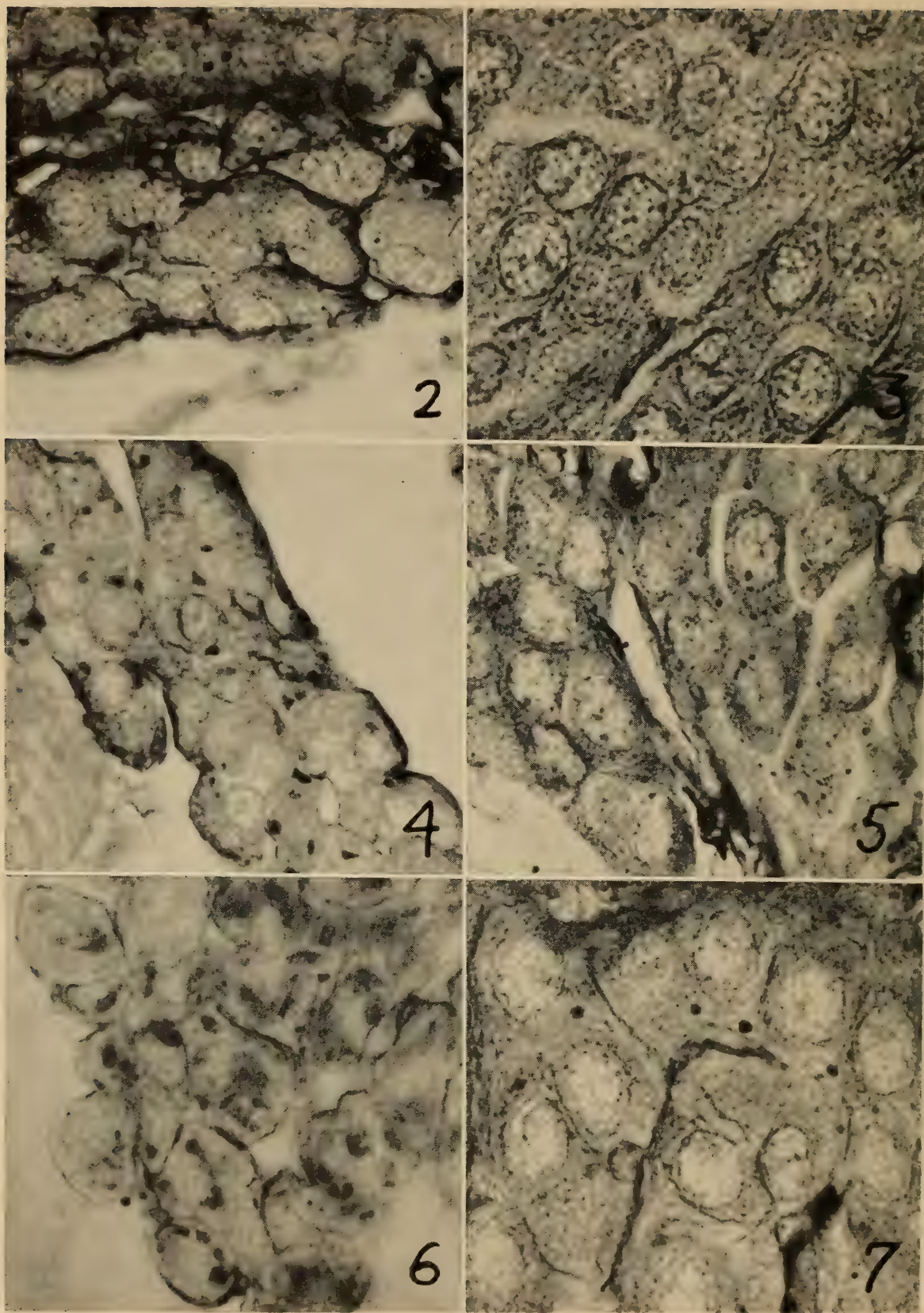
For *Gecarcinus*, Bliss (1956) reported that shortly before the animal molts growth occurs again. She termed this period *premolt growth*. Only in some instances was it possible to detect this period in the young *Orconectes* used in the present study. In a few animals a suggestion of the premolt growth stage was detected (Fig. 1, animals A and B). Since this period is often short, it is possible that it occurred between the last measurement and the molt of the animal. With a measurement interval of 3–5 days, therefore, it would be possible to detect in most cases only a slight increase, if any, in the rate of limb regeneration at the end of the period of plateau. In view of this possibility, it is interesting that some animals which were caused to autotomize passed through a period of basal growth with the normal slope. However, these animals did not show a period of plateau (Fig. 1, animals D and E). Instead, growth continued at a rapid rate, and the animals molted within a few days. It is believed that these animals had been collected very near the end of their intermolt period and that basal growth and premolt growth are continuous when autotomy occurs late in the intermolt period.

In summary, it is seen that the regeneration of autotomized limbs follows the same pattern in *Orconectes* as in *Gecarcinus*. Increase in length is restricted to the periods of basal growth and premolt growth. Since the slope of the basal growth curve remains the same at different stages in the molt cycle, it appears that this stage is not directly under endocrine control. On the other hand, premolt growth apparently is influenced by endocrine changes in the animal. Evidence for this is found later in this paper and in the study by Bliss (1956).

Y organ

A non-neurosecretory endocrine gland which functions in the control of growth in crustaceans is the y organ. Originally described by Gabe in many species of crustaceans (1953), it is located in the second maxillary segment in *Orconectes limosa*. It is always closely associated with the base of one of the large mandibular muscles and is close to, but not in contact with, the hypodermis. In serial cross-sections it lies dorso-laterally to the circumoesophageal connectives and posterior to the oesophagus. The y organ of this animal is irregular in shape; its location and rambling structure would make it difficult to extirpate.

Histologically the cells of the y organ (Figs. 2, 4, and 6) are fairly uniform in size, and very fine strands of connective tissue divide the organ into short irregular cords about 1–2 cells in thickness. Blood spaces appear in the organ but are not particularly numerous. Frequently, however, blood cells can be seen between the cords of cells. The cells of the y organ possess a very finely granular, slightly acidophilic cytoplasm with well defined cell boundaries. The nucleus is sharply outlined and usually contains a single nucleolus. Scattered in the cytoplasm of the cells the secretory material appears in the form of irregular clusters of very small aldehyde fuchsin-positive granules. When the secretory material is present in very large amounts, the cytoplasm in general is stained with aldehyde fuchsin.



FIGURES 2-7.

At these times granules or large droplets of secretory material usually are present also.

During the greater part of the intermolt period, the cells of the y organ contain moderate amounts of aldehyde fuchsin-positive material (Fig. 4). Apparently all, or almost all, of the cells possess similar amounts of secretory material at any particular time.

At molt, however, the y organ undergoes striking changes in its secretory content. Animals fixed during the first four or five days after shedding possess y organs in which it has not been possible to detect secretory material with the aldehyde fuchsin technique. The cytoplasm of these cells is apparently empty of secretory material (Fig. 2). The disappearance of the secretory material is definitely correlated with the molt of the animal although the two may not occur simultaneously. Some animals, but not all, possess empty y organs before molt. All animals possess empty y organs immediately following molt. In two cases, animals fixed while actually in the shedding process had y organs which contained more secretory material than normally (Fig. 6).

It is interesting to note that, while all animals possess secretory granules during most of the intermolt period, it is not possible to determine from y organ examination alone how near to an approaching molt the animals are. Careful study of serial sections of y organs does suggest, however, that the size of the granules in the y organ cells increases as the animals approach molt.

Neurosecretory cells

In Table I are recorded the counts of the type 2 neurosecretory cells which contain secretory material in the x organs of animals fixed at different times during the intermolt period. It is obvious that a large number of cells possess secretory material at all times except molt. Thus, from 1–2 days preceding molt to 4–5 days after molt very few type 2 cells contain secretory droplets or granules (Fig. 3). Indeed, most of the type 2 cells are devoid of all signs of secretory activity during this period. Many type 2 cells contain granules or droplets of secretory material from a few days following molt to just before the next molt (Figs. 5 and 7).

Of further interest is the observation that shortly after molt, when secretory

FIGURE 2. Y organ from an animal during the first few days after molt. Secretory material is absent from the cells. Bouin plus calcium chloride; aldehyde fuchsin; 1140 $\times$.

FIGURE 3. Type 2 neurosecretory cells in the x organ of an animal on the day after molt. Secretory material is absent from the cells. Bouin plus calcium chloride; aldehyde fuchsin; 1140 $\times$.

FIGURE 4. Y organ from an animal in the basal growth stage of limb regeneration. The cells contain only a moderate amount of secretory material. Bouin plus calcium chloride; aldehyde fuchsin; 1140 $\times$.

FIGURE 5. Type 2 neurosecretory cells in the x organ of an animal in the basal growth stage of limb regeneration. The secretory material in these cells as clumps of very small granules. Bouin plus calcium chloride; aldehyde fuchsin; 1140 $\times$.

FIGURE 6. Y organ from an animal during the molt process. This animal possessed a high type 2 cell count. Note the large amount of secretory material in the cells. Bouin plus calcium chloride; aldehyde fuchsin; 1140 $\times$.

FIGURE 7. Type 2 neurosecretory cells in the x organ of an animal during the plateau stage of limb regeneration. Note that the secretory material occurs in the form of droplets. Bouin plus calcium chloride; aldehyde fuchsin; 1140 $\times$.

material is again present in the type 2 cells, the material is in the form of numerous small granules scattered throughout the cytoplasm of each cell. Later in the intermolt period the secretory material is present in the form of fewer and larger droplets (Table I and Figs. 5 and 7), similar to those observed in *Orconectes*

TABLE I
*Counts of type 2 neurosecretory cells which contained secretory material
in the x organ*

Animal number	Stage of molt cycle	Type 2 cell count	Form of material	
			Droplets	Granules
3	molting	92	+	—
39	molting	50	+	—
1	molt + hrs.	0	—	—
5	molt + hrs.	19	+	—
6	molt + hrs.	0	—	—
2	molt + 1 d.	0	—	—
58	molt + 1 d.	3	—	—
7	molt + 4 d.	25	+	—
8	molt + 4 d.	34	+	—
23	molt + 4 d.	46	+	—
24	molt + 5 d.	83	+	—
37	basal gr.	114	+	—
10	basal gr.	35	—	+
11	basal gr.	51	—	+
25	basal gr.	85	+	+
40	basal gr.	27	—	+
41	basal gr.	80	—	+
12	early plat.	70	+	—
14	early plat.	118	—	+
15	early plat.	126	—	+
17	early plat.	98	—	+
26	early plat.	70	—	+
29	early plat.	62	+	—
30	early plat.	47	+	—
31	early plat.	75	+	—
32	early plat.	74	+	—
33	early plat.	23	+	—
18	late plat.	57	+	—
19	late plat.	117	+	+
20	late plat.	66	+	—
21	late plat.	64	+	—
22	late plat.	51	+	—
42	late plat.	49	+	—
46	late plat.	31	+	—
43	premolt gr.	10	+	—
44	premolt gr.	20	+	—
45	premolt gr.	6	+	—

virilis. In an earlier study (Durand, 1956) it was suggested that the presence of secretory material in the form of small granules might represent an early stage in the production of the material. If this is so, then the absence of any signs of secretory material in the x organ type 2 neurosecretory cells in the period immediately follow-

ing molt would indicate that the synthetic activities of these cells had ceased. It is during this time that many active growth processes occur.

DISCUSSION

So far we have seen that major changes occur in the two endocrine organs, x organ and y organ, at the time the animal molts. The secretory activity of the x organ neurosecretory cells, as indicated by type 2 cell counts, is compared to growth of regenerating limbs in Figure 8. The figure represents a generalization of the data, and an explanation of its construction is given in the next paragraph.

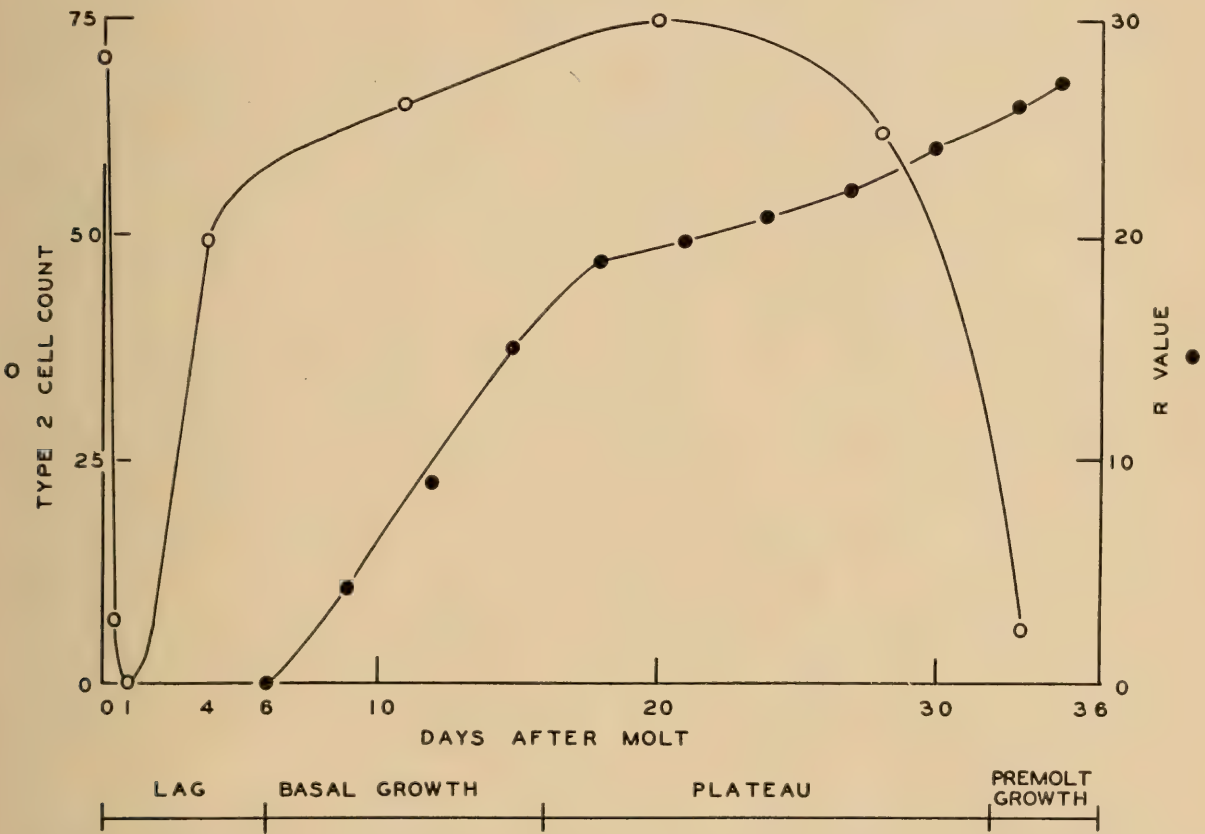


FIGURE 8. Type 2 neurosecretory cell counts and R values of crayfish with regenerating limbs at the indicated times during the period of regeneration.

After animals with regenerating limbs had molted, the R values for each animal were plotted against the per cent of the growth period, and smooth curves were drawn through the points. The *growth period* in this case included the time from the first appearance of a regenerating limb to the molt of the animal. From each of the curves, R values were obtained at 10% intervals of the growth period. If the mean lag period of 6 days is subtracted from the mean intermolt period of 36 days, each 10% interval in the growth period is equivalent to three days of the mean intermolt period. In Figure 8 the mean R values are plotted at each three-day interval. Also plotted are the type 2 cell counts from Table I. The mean length of time required for the completion of basal growth was 10-11 days, and the type 2 cell count for that stage was placed in the middle of that

time, on day 11 of the intermolt period. Days 16 to 32 would be the mean length of the plateau stage of limb regeneration if four days are allowed for the period of premolt growth. The mean type 2 cell counts for early and late plateau, therefore, were placed on day 20 and on day 28 of the intermolt period. These last two points represent, respectively, 25% and 75% of the plateau period. As discussed earlier, the premolt growth stage probably occurred in the last 3–5 days of the intermolt period, and so the type 2 cell count for that stage was placed on day 33, three days before molt.

Figure 8 shows that some animals possessed low type 2 cell counts during the premolt growth stage of limb regeneration and just after shedding. No high type 2 cell counts were found in animals during the first four days after shedding. In fact, the counts actually dropped to zero within 24 hours of shedding. About 4–5 days after molt the secretory material could be detected again in the perikaryon of the cells. Two notable exceptions are animals number 3 and number 39 (Table I). These animals were fixed while shedding but, nevertheless, possessed high type 2 cell counts. It should be pointed out that the y organs of these two animals, unlike those of other animals at molt, possessed unusually large amounts of secretory material. Apparently the initiation of the shedding process was independent of the y organ and x organ secretory content.

The y organ also underwent its greatest change in secretory content just prior to the molt of the animal. Thus, secretory granules were present in the y organ cells during most of the intermolt period except for a few days before and after molt. In addition the secretory granules disappeared from the y organ cells before the decrease in the type 2 cell count occurred. Thus, premolt animals were found to have high type 2 cell counts and full y organs, high type 2 cell counts and empty y organs, low type 2 cell counts and empty y organs. However, *no* animals were found to have low type 2 cell counts and full y organs.

The two endocrine organs consequently exhibited cyclical secretory behavior associated with molt. When their functions are considered, it is not surprising that they should do so. What is surprising is that both organs contained secretory material at approximately the same times during the molt cycle. On the basis of histological observations alone, it is difficult to interpret these results. For example, the presence of stainable material within a cell can mean either that the cell is producing and releasing its secretory products or that it is inactive. In the succeeding paragraphs certain physiological information concerning the two organs will be presented which suggests a possible interpretation of the observations reported here.

It is known that neurosecretory cells in the x organ produce a molt-inhibiting hormone in crabs (Passano, 1953). This hormone is passed via the neurosecretory cell axons to the sinus glands where it is stored for later release into the blood (Bliss, 1953; Bliss, Durand and Welsh, 1954; Carlisle, 1953; Passano, 1953). An earlier study by the author suggested that the type 2 neurosecretory cells in the x organ are the source of the molt-inhibiting hormone in the crayfish since they are the only neurosecretory cells that show histologically demonstrable quantitative changes in secretory material at molt. It was also suggested that the disappearance of secretory material from the type 2 cells could be accounted for by a rapid transfer of the material from the x organ to the sinus glands at molt. Another hypothesis is

that the rate of synthesis of the hormone was reduced below the rate of transfer. Since the type 2 cells are probably the source of the molt-inhibiting hormone and since the implantation of eyestalks inhibits the premolt growth stage of limb regeneration in crabs (Bliss, 1956), it is logical to assume that the presence of stainable material in the type 2 cells, during the intermolt period when molt-inhibiting hormone hypothetically is released, indicates that these cells are actively synthesizing the hormone. The absence of stainable material when molt-inhibiting hormone supposedly is withheld just before molt could result from a decreased rate of synthesis or an increased rate of transfer from the cell bodies to the sinus glands. The reappearance of the secretory material in the form of many small granules 4–5

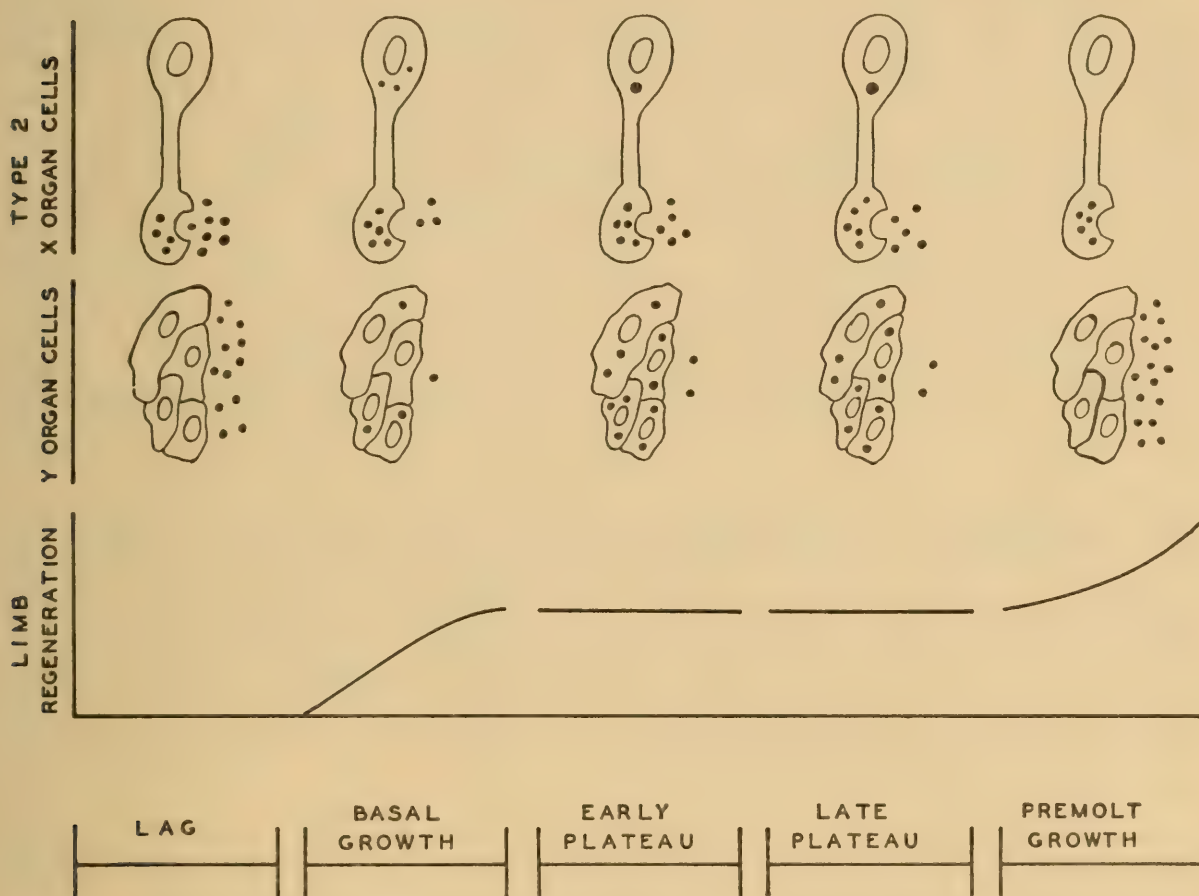


FIGURE 9. Diagrammatic summary of the secretory activity of x organ type 2 neurosecretory cells and the y organ cells in relation to limb regeneration and the molt cycle.

days after molt would result from a rate of synthesis greater than the rate of transfer from the perikaryon. Unfortunately, no information is available concerning the secretory content of the sinus glands during the stages studied in this investigation. However, Pyle (1943) has shown that the sinus glands of *Cambarus virilis* (now *Orconectes virilis*) lose most of their stainable material soon after molt.

Similar reasoning with respect to the condition in the y organ cells suggests that these cells may be actively secreting their hormone when they are histologically empty. Thus, it has been shown that the y organ is the source of a growth-promoting hormone in *Carcinides* by Echali er (1954, 1955). Travis (1955, 1957)

has shown that in *Panulirus* the resorption of the old integument occurs chiefly during the last three days before molt and the first two after molt. The production of all layers of the integument in *Panulirus* is completed by about eight days after molt. Travis reported that the intermolt period for the animals she used was 65–70 days. If these processes required a proportionate period of time in *Orconectes*, they would have occurred entirely during the period in which the y organ cells were devoid of their secretory products. For these reasons it seems logical to assume that the y organ cells are secreting their hormone when they are histologically empty at molt. However, we cannot conclude this until more is known about the physiology of the y organ and its interaction with the x organ cells.

In summary, an hypothesis can be constructed (Fig. 9) which agrees with the known facts but which requires additional experimental evidence. During the greater part of the intermolt period, secretory granules can be demonstrated in the type 2 neurosecretory cells and in the y organ cells. *Hypothetically*, molt-inhibiting hormone is released from the type 2 neurosecretory cells during this time while growth-promoting hormone is withheld by the y organ cells or released in insignificant quantities. The basal growth and plateau stages of limb regeneration can occur during this period. Just before molt and for a short time after, secretory material cannot be demonstrated in either the type 2 neurosecretory cells or the y organ cells. *Hypothetically*, the molt-inhibiting hormone of the x organ is not released at this time but the growth-promoting secretion of the y organ is released. At this time both premolt growth of regenerating limb buds and molt can occur.

SUMMARY

1. Four periods in the regeneration of autotomized limbs can be identified in the young of *Orconectes limosa*. The first, designated *lag period*, is one in which no growth occurs and lasts about 6 days. The second, the period of *basal growth*, is characterized by rapid growth of the regenerating limb. At the end of this period the regenerating limb is about 22% of the carapace in length. During the third period, called *plateau*, no growth occurs; this period occupies the greater part of the intermolt period. In the fourth period, *pre-molt growth*, rapid growth again occurs about 3–5 days before molt.

2. The y organ and its secretory behavior are described. Changes in the activity of the y organ can be demonstrated histologically for only a brief period which extends from about three days before molt to 4–5 days after molt.

3. The changes in the activity of the type 2 neurosecretory cells in the x organ are described. The most marked changes occur only just before molt and persist for a period of 4–5 days after molt.

4. The secretory activity of the x and y organs are discussed in relation to the molt cycle and the regeneration of autotomized limbs. Arguments are presented in favor of the hypothesis that the y organ cells produce and release their secretory products when the type 2 neurosecretory cells in the x organ are inactive.

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STUDIES ON THE STRUCTURE AND PHYSIOLOGY OF THE FLIGHT MUSCLES OF BIRDS. 9. A QUANTITATIVE STUDY OF THE DISTRIBUTION PATTERN OF SUCCINIC DEHYDROGENASE IN THE PECTORALIS MAJOR MUSCLE OF THE PIGEON

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George and Naik (1958a, 1958b) have shown that the *pectoralis major* muscle of the pigeon consists of two distinct types of fibers, a broad glycogen-loaded white variety with few or no mitochondria and a narrow fat-loaded red variety having a large number of mitochondria in them. George and Scaria (1958) in a histochemical study of dehydrogenases (succinic, malic, lactic, and glycerophosphate dehydrogenases) in this muscle could not detect the presence of any of these dehydrogenases in the broad white fibers. They therefore concluded that these fibers in the pigeon breast muscle are a unique system in which none of the oxidative processes concerned with the above enzymes takes place and therefore cannot be considered as analogous to the white fibers of the other vertebrate skeletal muscles studied. These observations should naturally raise some important questions as well as doubts regarding the exact role of the broad fibers in this muscle and also the problem of energetics for their contraction. But, however, the above conclusions were based solely on histochemical observations and can be considered decisive only if supported by quantitative studies. Here we report the results of a quantitative study of the distribution pattern of the succinic dehydrogenase activity in the *pectoralis major* muscle of the pigeon.

MATERIAL AND METHODS

It is technically impossible by ordinary methods to estimate directly the activity of any enzyme in the two types of fibers in the pigeon breast muscle after isolating the fiber types separately. But the indirect method, *viz.* of estimating the activity of the enzyme in different layers of the muscle, where the broad and narrow fibers vary in number, was adopted in the present study. The fiber architecture of the *pectoralis major* muscle of the pigeon has been worked out in detail by George and Naik (1959). They have found that the least number of broad fibers is found in the fasciculi situated in the middle of the dorso-ventral axis of the muscle and the number tends to increase above or below this mark, reaching the maximum in the most superficial and in the deepest layers of the muscle. They also showed that per unit area of cross-section of the muscle at any particular depth, the number of broad fibers is inversely proportional to the number of narrow fibers, and derived the formula

$$Y = - 5.75 X + 890.01,$$

(where Y stands for the number of narrow fibers and X for the number of broad fibers) in order to estimate the number of narrow fibers in any one particular square mm. area, X being known. Throughout this study we made use of this formula for finding out the number of narrow fibers in a particular layer of muscle which was used for the estimation of the succinic dehydrogenase activity.

The method employed in the estimation of succinic dehydrogenase was the one according to Kun and Abood (1949) which makes use of the principle that colorless TTC (2,3,5-triphenyl tetrazolium chloride) is reduced to a red insoluble com-

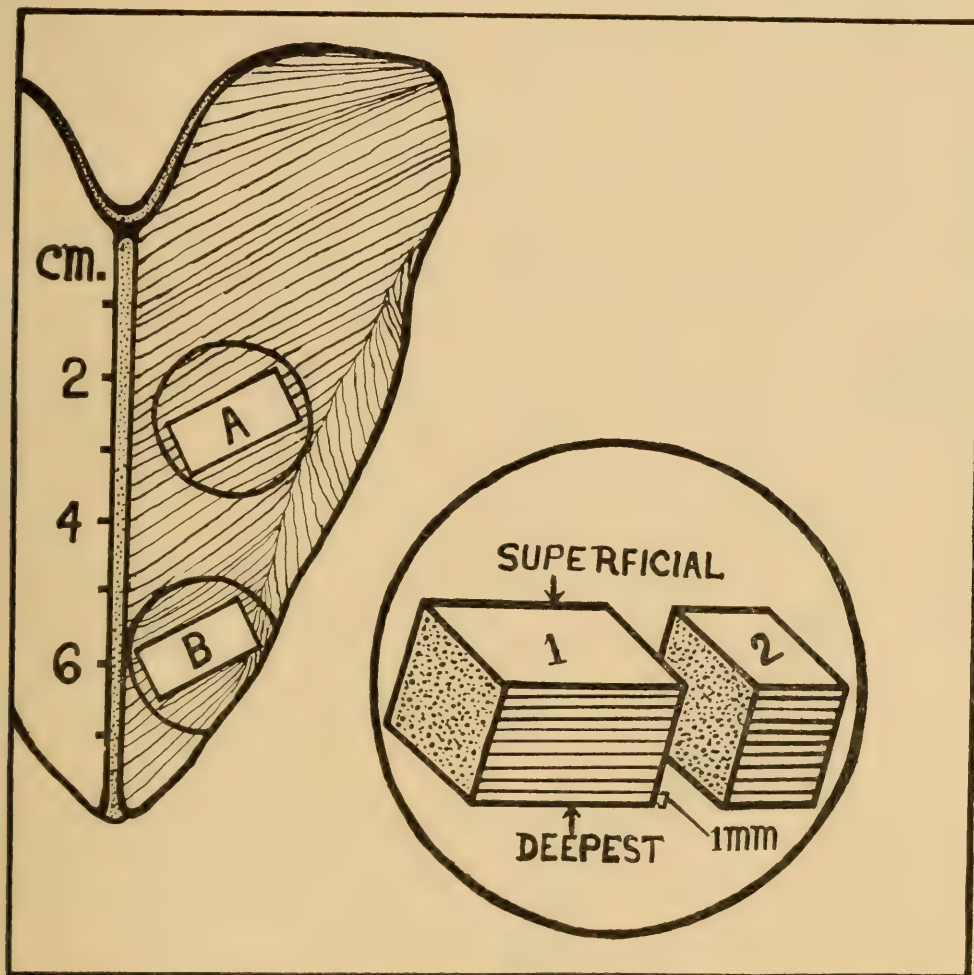


FIGURE 1. Illustrating the topography of the muscle pieces used for the study of succinic dehydrogenase activity in the pigeon breast muscle.

pound, formazan, by the enzymic oxidation of succinate to fumarate. The color developed was extracted in acetone and the intensity measured in a colorimeter. The amount of formazan formed was directly read from a standard curve plotted by reducing completely known quantities of TTC by sodium hydrosulphite. Since the reduction of TTC by hydrosulphite was found to be reversible, the color was stabilized by adding acetone within one minute of the addition of hydrosulphite to the TTC solution, during which time the TTC was completely reduced.

In order to ensure that only uniformly well developed *pectoralis major* muscle

was used in the study, well fed and fully grown, normal laboratory pigeons (*Columba livia*) were used throughout.

In each experiment a bird was decapitated and the blood completely drained off. Pieces of muscle from the regions shown in Figure 1 (A and B) were cut off for each set of experiments. A portion (1) of this muscle piece A was immediately mounted on the stage of a freezing microtome with the superficial (ventral) side facing up, so as to obtain serial horizontal sections of the desired thickness starting from the superficial side of the muscle. The remaining portion (2) of the same piece was set apart for counting the broad and narrow fibers. The same procedure was followed for muscle piece B. In most cases it was found necessary to freeze the muscle block first with the superficial (ventral) face down and after trimming the deeper (dorsal) side it was reversed so as to render the surface even and parallel to the edge of the knife. When the block was frozen hard the epimysium was removed by a superficial stroke of the microtome knife and serial sections of 0.5 mm. or 1.0 mm. thickness as required, were cut. The sections were immediately transferred to weighing bottles and the weight determined. They were then chilled and each of the sections was homogenized in 10 ml. of distilled water. The succinic dehydrogenase activity of this homogenate was then determined using the method mentioned above.

The incubation mixture in each case contained 0.5 ml. of 0.1 *M* phosphate buffer of pH 7.4, 0.5 ml. of 0.2 *M* sodium succinate, 1.0 ml. of the muscle homogenate and 1.0 ml. of a freshly prepared 0.1% solution of TTC in a total volume of 3 ml. The TTC solution was added last, and after shaking, the tubes were incubated for 15 minutes at 37° C. Seven ml. of acetone were then added to each tube. The tubes were tightly stoppered, shaken well and centrifuged for about three minutes at about 3000 r.p.m. The clear supernatant was drawn off and the intensity of the color read at 420 $m\mu$ on a Klett-Summerson photoelectric colorimeter. The readings were corrected for a blank with zero time incubation. Inhibition experiments with malonate confirmed that the color production was solely due to succinic dehydrogenase.

Part (2) of the original piece of the muscle was sectioned as shown in Figure 1. The section was mounted in 50% glycerine and the number of broad fibers per square mm. counted according to the method described by George and Naik (1959). From the broad fiber count, the number of narrow fibers in the particular area was calculated using the formula already referred to.

RESULTS

The dehydrogenase activity is expressed in the number of micrograms of formazan produced by 100 mg. wet weight of the muscle for 15 minutes at 37° C. under the conditions of the experiment mentioned above. We do not claim that the values given are absolute because it is likely that freezing and thawing of the tissue in the process of cutting sections destroys a certain amount of its enzyme activity. However, this loss of activity could not be considerable and could be even neglected in view of the fact that our purpose is to compare the activity at the different depths and in relation to the number of the fibers per square mm. area.

The succinic dehydrogenase activity and the number of broad and narrow fibers per square mm. in the different layers of the regions A and B of the breast

TABLE I

Showing the succinic dehydrogenase activity in the different layers of the regions A and B (marked in Fig. 1) of the pectoralis major muscle of the pigeon and its relation to the number of narrow fibers. (The figures indicate the average values of five sets of readings.)

Exp.	Depths of the muscle in mm. starting from the superficial side (ventral face)	No. of broad fibers per square mm.		No. of narrow fibers per square mm.		Succinic dehydrogenase activity in μ g. of formazan per 100 mg. of wet muscle per 15 minutes $\pm$ S.D.	
		Region A	Region B	Region A	Region B	Region A	Region B
1	0-0.5	132	120	131	200	15.5 $\pm$ 2.17	23.0 $\pm$ 1.18
	0.5-1.0	100	98	315	327	35.5 $\pm$ 1.98	40.0 $\pm$ 1.56
2	0-1	107	101	278	317	32.22 $\pm$ 1.12	36.75 $\pm$ 3.13
	1-2	90	59	372	558	42.72 $\pm$ 1.37	64.47 $\pm$ 1.42
	2-3	86	50	402	609	46.25 $\pm$ 1.44	69.37 $\pm$ 0.82
	3-4	75	43	465	646	53.75 $\pm$ 1.15	75.87 $\pm$ 0.54
	4-5	59	40	549	662	63.25 $\pm$ 1.73	78.77 $\pm$ 1.32
	5-6	45	48	638	617	72.50 $\pm$ 1.05	70.00 $\pm$ 1.08
	6-7	43	52	648	595	72.80 $\pm$ 0.92	68.50 $\pm$ 2.16
	7-8	41	58	658	564	74.50 $\pm$ 1.38	65.00 $\pm$ 1.45
	8-9	42	66	652	517	73.40 $\pm$ 2.10	59.00 $\pm$ 2.80
	9-10	45	76	635	457	70.00 $\pm$ 1.78	53.00 $\pm$ 2.55

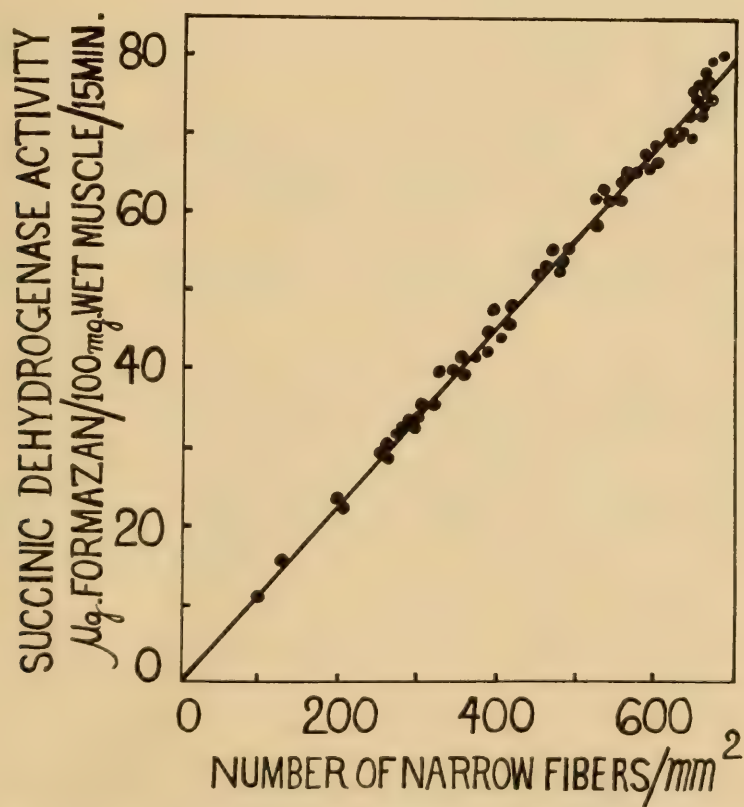


FIGURE 2. Graph showing the relation between the number of narrow fibers and succinic dehydrogenase activity in the different layers of the pectoralis major muscle of the pigeon.

muscle of the pigeon is shown in Table I. The results indicate that there exists a relationship between the activity of the enzyme and the number of narrow fibers per square mm. in the different layers of the muscle. It was also decided to find out the activity of the enzyme in the 0.5-mm. thickness of the most superficial layers of A and B where the broad fibers are relatively much more concentrated than in the one-mm. thick layer (Table I).

From the results obtained the regression line is drawn using the equation

$$S = 0.3 + .11478 Y,$$

where S stands for the succinic dehydrogenase activity and Y for the number of narrow fibers in one square mm. area. If the value of Y is zero the value of S

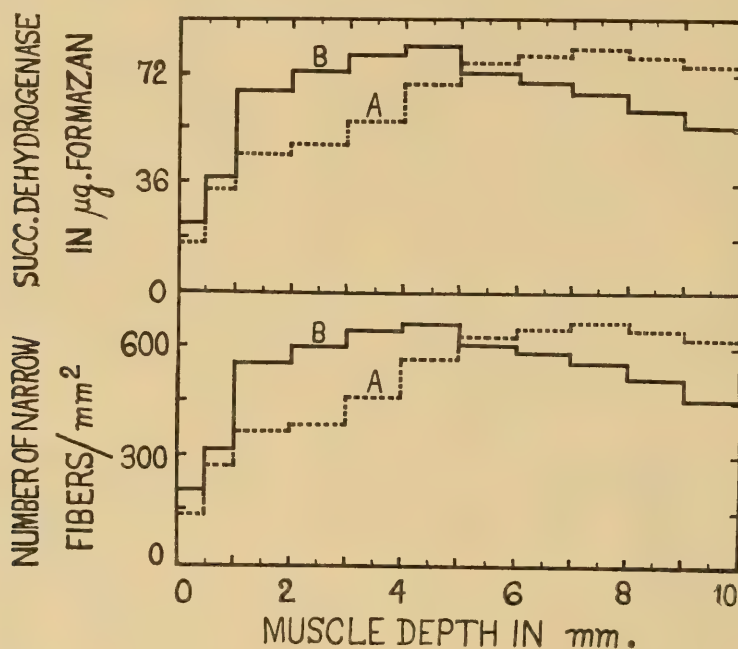


FIGURE 3. Showing the number of narrow fibers and the corresponding succinic dehydrogenase activity in the different layers of the two regions A and B of the *pectoralis major* muscle of the pigeon.

will be 0.3 μ g. of formazan, which is an infinitely small measure of activity. If the values obtained for the succinic dehydrogenase activity are plotted against the number of narrow fibers per square mm. a linear graph illustrating this relationship is obtained using the equation of the regression line (Fig. 2). Similarly plotting the dehydrogenase activity and the number of narrow fibers in the different layers from regions A and B almost a similar pattern is obtained (Fig. 3). In the most superficial region, which consists mostly of broad fibers, the succinic dehydrogenase activity more or less corresponds to that of the number of narrow fibers.

DISCUSSION

From the data presented above it appears that there exists a relationship between the number of narrow fibers and succinic dehydrogenase activity in the different layers of the pigeon breast muscle. This relationship can be traced to the fact that

the activity of the enzyme in the broad fibers is negligible. By substitution of the values in the formula mentioned above we obtain

$$S = 0.11478 (890.01 - 5.75 X),$$

(where S stands for the succinic dehydrogenase activity in $\mu\text{g.}$ of formazan per 100 mg. of wet weight of the muscle taken from any region parallel to the surface of the muscle, per 15 minutes under the conditions of our experiment mentioned earlier, and X stands for the average number of broad fibers per square mm. area of cross-section of the muscle). In other terms

$$\frac{S}{(890.01 - 5.75X)} = K,$$

(where K is a constant, the value of which is 0.11478). This indicates that S in any particular layer of the pigeon breast muscle is a function of the number of narrow fibers. The conclusion that can be drawn from this study is that in the pigeon breast muscle the main bulk of the succinic dehydrogenase is confined to the narrow fibers and that the broad white fibers possess only a negligible concentration of the enzyme.

George and Scaria (1958) and George, Susheela and Scaria (1958) studied, using histochemical methods, the activity of certain dehydrogenases in the breast muscle of the pigeon, fowl and bat and the leg muscle of the fowl and frog, and observed that the concentration of these oxidative enzymes has a relationship with the color and the mitochondrial content of the individual muscle fibers in the sense that the red narrow fibers possess a high concentration of these enzymes and mitochondria, in sharp contrast to the broad white fibers. Similar observations were made by Nachmias and Padykula (1958) in the skeletal muscle of rat. It should be pointed out here that within the limitations of the methods employed in all the above mentioned muscles, all the fibers in every muscle, except the broad ones of the pigeon breast muscle, dehydrogenase activity was detectable. Nevertheless the activity was varying in the different muscle fibers according to their diameter, highest activity being in the smallest fibers. In the pigeon breast muscle, however, where the same histochemical methods were employed the broad white fibers did not show the presence of any of the dehydrogenases mentioned earlier (George and Scaria 1958). The quantitative data presented in this paper show the distribution pattern of the succinic dehydrogenase activity in the different layers of the pigeon breast muscle and also that the main bulk of the enzyme is confined to the narrow red fibers.

SUMMARY

The relative distribution pattern of the narrow red and broad white fibers, and the succinic dehydrogenase activity in the different layers of the pigeon breast muscle were studied quantitatively. It was found that the activity of this enzyme in any particular layer of the muscle is more or less related to the number of the narrow red fibers present there since the main bulk of the enzyme resides in these fibers.

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THE EFFECTS OF OXYGEN POISONING ON THE POST-EMBRYONIC DEVELOPMENT AND BEHAVIOR OF A CHALCID WASP¹

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Aerobic organisms survive only within a narrow range of oxygen tensions. In very low oxygen, respiration becomes impossible; in excessively high oxygen tensions, oxygen poisoning occurs. This report describes the effect of high pressures of oxygen on the development and behavior of the chalcid wasp, *Mormoniella vitripennis*. Few higher organisms are as suitable subjects for studies of oxygen poisoning as insects. The design of their respiratory and circulatory systems is such that insects are less prone to carbon dioxide accumulation and aeroembolism ("bends") than vertebrates. The small size of *Mormoniella* also tends to minimize such complications. Further, *Mormoniella* is a particularly attractive experimental animal because the histological details of its post-embryonic development are well known (Tiegs, 1922).

In preliminary notes (Goldsmith and Schneiderman, 1956, 1958) we reported that the sensitivity of *Mormoniella* to oxygen poisoning changed in a systematic way during post-embryonic life. The present paper describes these changes in detail and identifies the organ systems which are the main targets of oxygen poisoning at successive stages in the life history.

MATERIALS AND METHODS

1. *Experimental animals*

Mormoniella vitripennis Walker is parasitic on pupae of muscoid flies. Tiegs (1922) has described the developmental anatomy of this wasp; Whiting (1955), and Schneiderman and Horwitz (1958) have recently reviewed its life-history. The adult female pierces the puparium and lays her eggs on the developing fly pupa. A few days later the eggs hatch and the larvae begin feeding on the host. The cells of a larva grow in size throughout the several instars, but according to Tiegs, there appears to be no larval cell proliferation. At 25° C. a larva feeds for about four days and molts several times but is unable to defecate. Finally, it ceases feeding and enters a resting stage during which the larval tissues begin to break down; simultaneously the imaginal discs proliferate. After only a few cell divisions the thin partition between the midgut and the invaginated rectum breaks down, defecation occurs, and the greyish larva becomes white. During the 24 hours immediately before and after defecation, the so-called prepupal period, the

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larval cells break down and the cell divisions necessary for the formation of the adult integument, appendages, nervous system, and certain muscles occur. Except for the thoracic and abdominal muscles, whose myoblasts continue dividing for about 15 hours after pupation, adult development is a period of refinement, molding, and differentiation of tissues and organs into the final imaginal form.

A time-table, descriptive of the development from egg to adult, is given in Figure 1. Following Snodgrass (1935) and Hinton (1946, 1948, 1958), an instar is considered ended when the epidermis retracts from the cuticle. In *Mormoniella*, hours or days may intervene between the time of detachment and the actual ecdysis, which unveils the new instar. Thus the pupal stage actually begins just prior to defecation when the cuticle of the last instar larva separates from the epidermis (evidenced externally by wrinkles). The pupa itself is not

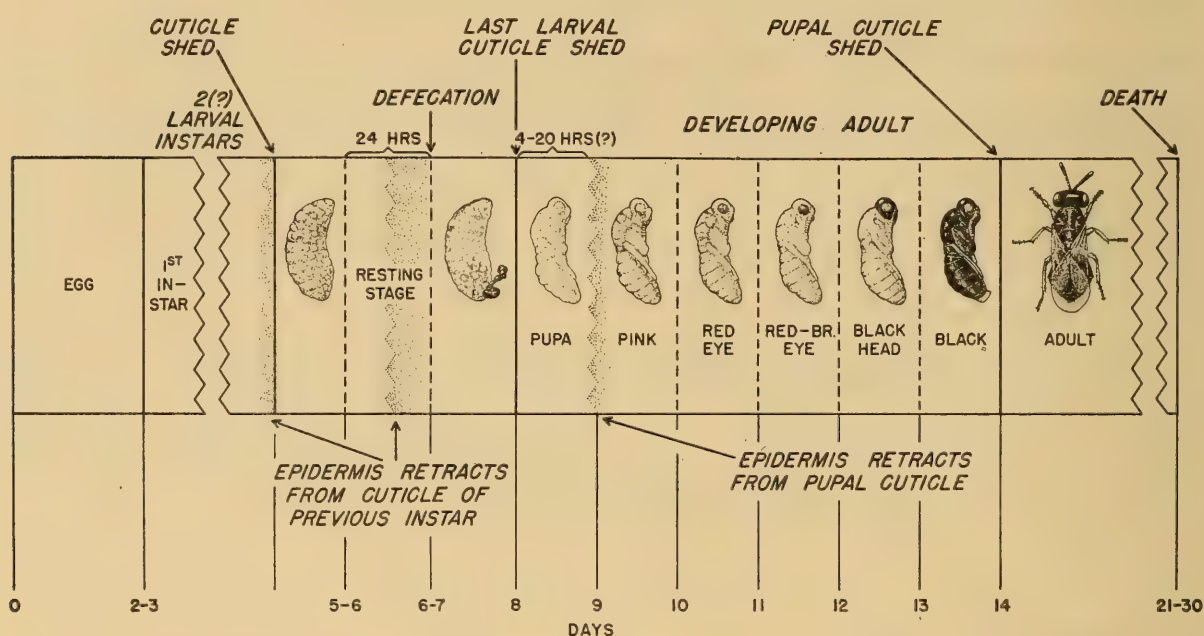


FIGURE 1. Time-table for the development of *Mormoniella*. The approximate times at which the cuticle detaches from the epidermis (signaling the end of an instar) are indicated by stippling.

revealed until the cuticle of the last larval instar is shed, an event occurring 24 to 48 hours later. The so-called prepupa is simply the pupa enclosed within the last larval cuticle. Likewise, the pupal stage ends with the detachment and retraction of the pupal cuticle, although the adult does not emerge for 4 or 5 days. During this period the wasp, which is properly called a developing adult, is enclosed in two cuticles—an inner adult cuticle and an outer detached pupal cuticle, which is shed at eclosion. The exact time at which epidermis retracts from the pupal cuticle, marking the end of the pupal period, has not been definitely determined for *Mormoniella*. Our observations agree with those of Tiegs in suggesting that this occurs within 24 hours after shedding the final larval cuticle.

Under certain conditions, just prior to entering the resting stage the larva ceases development and enters diapause (Schneiderman, 1957; Schneiderman and Horwitz, 1958). At room temperature diapause persists for a year or more

until the animal dies. If the diapausing larva is chilled at 5° C. for 3 months or longer, diapause ends and development resumes after return to 25° or 30° C. Chilled diapausing larvae may be stored in the cold for a year or more and provide a convenient source of experimental animals.

Mormoniella were reared at 30° C. on puparia of the fly *Sarcophaga bullata*. Since the wasp's development is accompanied by changes in epidermal pigmentation which are readily seen through its transparent pupal cuticle (Fig. 1), it was easy to select animals at specific stages of development and to observe the progress of development after experimental treatment. To assure that all insects used in a single experiment were of the same age and were developing at the same rate, only developing adults that reached a specified developmental stage within six hours of each other were used in experiments. Unfortunately larval development, unlike adult development, is not marked by gross external changes. Diapause, however, always occurs at the end of larval life; consequently, all diapausing larvae are at precisely the same stage of development. In the experiments to be described, larvae which had entered diapause were collected and stored at 5° C. for 4 to 8 months before use. These larvae initiated pupal development within 24 hours after return to 30° C. For the present investigation, animals at five stages of development were used: (1) chilled diapausing larvae (equilibrated at 30° C. for one hour); (2) early prepupae (chilled diapausing larvae placed at 30° C. for 24 hours prior to the experiment and thus just beginning pupal development); (3) "pink stage" developing adults (24 hours after ecdysis from the final larval cuticle); (4) "black stage" developing adults (12 to 24 hours prior to adult emergence); and (5) adults (males and females). In each experiment groups of about ten animals were placed in one-dram shell vials loosely plugged with cotton. Several vials were compressed in each pressure chamber. After exposure and decompression the vials were removed from the chambers and placed at 30° C., where the progress of development was observed periodically until the adults emerged or the insects died.

In every experiment, 25 to 50 insects were kept in air at atmospheric pressure during the experimental period. These air controls indicated the normal rate of development and percentage of adult emergence for a given population. The adult emergence of these control insects varied between 75 and 100 per cent. Therefore, following an experimental treatment only consistent reductions of 50 per cent or more in adult emergence were considered different from the control values. To permit a comparison of the effects of different experimental conditions, a number of groups of insects at the same stage of development were selected from the same population, and each group exposed simultaneously in separate chambers to specific experimental conditions. The data from each group were compared with those of other groups in the same experiment. Each experiment was repeated one to several times. The data presented in this paper are representative of that amassed from almost 10,000 animals studied in a total of about 350 separate compressions (Goldsmith, 1955).

2. Compression and decompression

Commercial cylinders of compressed oxygen, nitrogen, and helium (Airco or Matheson) were used. All gases contained less than 0.5 per cent impurities

(Schneiderman *et al.*, 1953). The vials of wasps were sealed in transparent polymethylmethacrylate (Lucite) chambers fitted with brass end-plates and needle valves (Schneiderman and Feder, 1954) and compressed with a specific gas. In all cases the gases were superimposed on the atmosphere of air initially in the chamber. Throughout this paper all pressures are given as gauge pressure. The final pressure in each chamber was checked on the same gauge, both after compression and before decompression, and the insects from any chamber showing greater than ± 5 per cent variation in pressure during the experimental period were discarded. The sensitivity of the gauges at the pressures employed was about ± 3 per cent.

Compression was accomplished in one minute, whereas decompression was performed stepwise over a period of five minutes. The pressure was reduced to half in the first minute of decompression and then held constant during the second minute. In the third minute, the pressure was gradually reduced to one fourth and then again held constant during the fourth minute. During the final minute, the pressure was gradually reduced to atmospheric.

The chambers were kept at a specific temperature in a thermoregulated water bath. Most experiments were conducted at $30 \pm 1^\circ$ C. and positive pressures (gauge) of 5 atmospheres (atms.) oxygen. Under these conditions, adult wasps survived for 1 or 2 hours. Hence, the time required for compression and decompression occupied only a small fraction of the total exposure time.

RESULTS

1. *The effect of pressure on Mormonella*

In each experiment, wasps at various stages of development were exposed to positive pressures of nitrogen or helium to determine if elevated pressures of metabolically inert gases had any toxic effects. As Table I shows, prolonged exposure for 16 hours to 5 atms. of helium had, at most, a slight effect on survival and development of any stage of the life cycle. Since 5 atms. of helium did not impair the development of *Mormoniella*, we may conclude that this pressure *per se* does not adversely affect *Mormoniella*. By comparison with either helium or nitrogen³ the toxicity of 5 atms. oxygen is striking. For example, in experiment No. 104, 78 per cent of the control chilled diapausing larvae ultimately emerged as adults. After 16 hours of exposure to 5 atms. helium, a somewhat smaller percentage emerged (70 per cent), but after the same exposure to oxygen only 13 per cent emerged.

2. *The effects of compression and decompression on Mormonella*

As Paul Bert (1878) first pointed out, the adverse effects of rapid decompression on vertebrates result in large measure from nitrogen being released from the tissues too fast to be dissolved. Since *Mormoniella* is small in size and possesses a tracheal system, rapid decompression might affect this insect differently from a vertebrate. To test this, a total of 1,440 chilled diapausing larvae, early prepupae,

³ The survival and development of insects exposed to 5 atms. nitrogen was somewhat more variable than that of insects subjected to helium, an effect which can be attributed to nitrogen narcosis (Frankel and Schneiderman, 1958).

TABLE I

Effect of exposing Mormoniella at specific stages of development to 5 atms. of He or O₂ at 30° C. for various periods

Expt. No.		Per cent emerging			
		104*	109*	114**	115**
Duration of exposure (hrs.) \ Stage of devel.		Chilled diapausing larvae	Early prepupae	"Pink stage"	"Black stage"
Air (controls at atm. pressure)		78	90	96	100
He	2	88	86	—	—
	4	94	85	—	—
	8	64	94	—	—
	16	70	82	—	100
	24	—	—	56	88
	32	—	—	84	100
	64	60	78	80	92
O ₂	1	72	51	—	—
	2	73	55	88	96
	4	70	54	80	94
	8	63	65	40	0
	16	13	20	0	—
	24	—	—	0	—
	64	—	0	—	—

* About 50 individuals used in each exposure.

** About 25 individuals used in each exposure.

"pink" and "black stage" developing adults were compressed with 10 atms. nitrogen and then decompressed. The following procedures were used to study the effects of rapid compression or decompression:

Group I: Controls—maintained at atmospheric pressure.

Group II: Thirty seconds for compression and thirty seconds for decompression.

Group III: Thirty seconds for compression and five minutes for decompression following the regimen outlined in the section on Methods.

Group IV: Five minutes for compression and five minutes for decompression.

In each group, two tanks were compressed and ten minutes elapsed between compression and decompression. Regardless of the regimen, wasps exposed after the start of the prepupal period invariably emerged as normal adults in about the same time as air controls. By contrast, chilled diapausing larvae proved exceedingly sensitive to compression. Though 85 per cent of air controls in this experiment completed adult development, fewer than 40 per cent of the chilled diapausing larvae emerged as adults when subjected to any of the above procedures. Because of the unusual sensitivity of this stage to compression and decompression, only one successful experiment was conducted with some 750 chilled diapausing

TABLE II

Effect of exposing Mormoniella prepupae to O₂ at 30° C. for various periods

Expt. No.....	49* (10 atms.)	115** (5 atms.)	116** (5 atms.)
Exposure (hours)	Per cent emerging as adults		
Air (control at atm. pressure)	85	85	82
O ₂ $\frac{1}{3}$	90	—	—
$\frac{2}{3}$	45	—	—
1	59	—	—
2	—	50	44
4	35	40	44
8	—	46	40
16	0	4	11
32	—	—	0

* Each figure is based on about 20 individuals.

** Each figure is based on about 25 individuals.

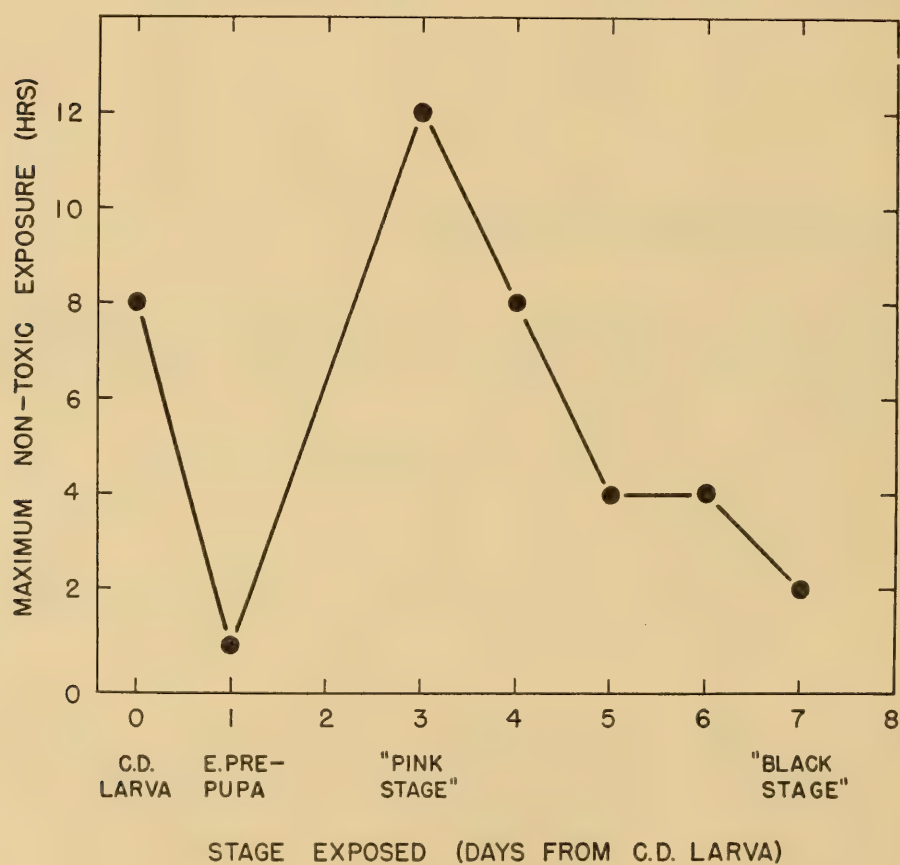


FIGURE 2. The sensitivity of different developmental stages of *Mormoniella* to 5 atms. O₂ at 30° C. The maximum exposure that had no effect on adult emergence is plotted as a function of developmental stage from chilled diapausing (C.D.) larva to "black stage." Stages withstanding only a brief exposure are the most sensitive to oxygen poisoning.

larvae (Exp. 104, Table I). In this experiment, for reasons which are not clear, the controls exposed to 5 atmospheres helium showed no evidence of an adverse effect of pressure or decompression and compression.

3. *Sensitivity of different developmental stages to oxygen poisoning*

We have established that, excepting chilled diapausing larvae, pressure as such, as well as compression and decompression, has only minor effects on the development of *Mormoniella*, and can now consider oxygen poisoning itself. In the first series of experiments we examined the effects of five atmospheres of oxygen on various developmental stages. The toxic effects of oxygen were appraised in terms of: (a) the rate of development of the wasps, (b) the percentage reaching maturity and emerging, and (c) the duration of exposure necessary to halt all development completely. Tables I and II give the effects of exposure to oxygen at several developmental stages on the ultimate emergence of the adults. Figure 3 and Table II record the final stage of development reached by wasps exposed to oxygen at a specific developmental stage.

To measure the inhibition of adult emergence, we determined for each developmental stage the maximal exposure time that had no effect on emergence. It is clear that this insect's sensitivity to oxygen changes markedly during development (Fig. 2). Chilled diapausing larvae and "pink stage" wasps were the least sensitive and tolerated more than 8 hours of oxygen. Early prepupae were many times more sensitive and could barely withstand one hour of oxygen. Thus, during the 24-hour period in which the chilled diapausing larva transformed into an early prepupa, sensitivity to oxygen increased many-fold. Likewise, after the "pink stage," sensitivity progressively increased during adult development, reaching its maximum in the adult wasp which could tolerate only one-sixth the exposure of a "pink stage" wasp.

4. *Oxygen poisoning in chilled diapausing larvae and early prepupae*

The above experiments disclosed that chilled diapausing larvae were far less sensitive to oxygen than early prepupae. To reduce adult emergence of chilled diapausing larvae by 50 per cent, an exposure to oxygen for 8 to 16 hours was required; for early prepupae, however, an exposure of less than one hour at 5 atms. must have been sufficient (Table I). Since at 10 atms. an exposure of 20 minutes had no effect on the subsequent emergence of prepupae (Table II, Experiment 49), it may be inferred that at 5 atmospheres of oxygen an exposure between 20 minutes and one hour would be required to reduce emergence by half.

Curiously, exposing early prepupae to 5 atms. oxygen for 2, 4 and even 8 hours had no greater effect on the subsequent emergence of adults than did one hour of exposure (Table I). Thus, although the early prepupa was the most sensitive developmental stage studied, this sensitivity was manifest in only half the population. The other half showed a sensitivity comparable to chilled larvae. These results were confirmed in numerous experiments with early prepupae (for example, Table II, Exp. 115 and 116). The explanation of this phenomenon is not clear; there is no marked difference in sensitivity of males and females, nor does this result stem from effects of compression or decompression. A likely explanation

is that the resistant members of the population were slower in initiating pupal development and at the time of exposure to oxygen were still in a stage comparable to the insensitive chilled diapausing larva.

Eight hours' exposure to oxygen caused a retardation in the rate of development of both chilled diapausing larvae and early prepupae; both lagged behind the controls by 1 or 2 days. Though adult emergence was reduced, prolonged exposure to oxygen failed to cause an arrest in development comparable to diapause. In fact chilled diapausing larvae and early prepupae defecated, and many continued to

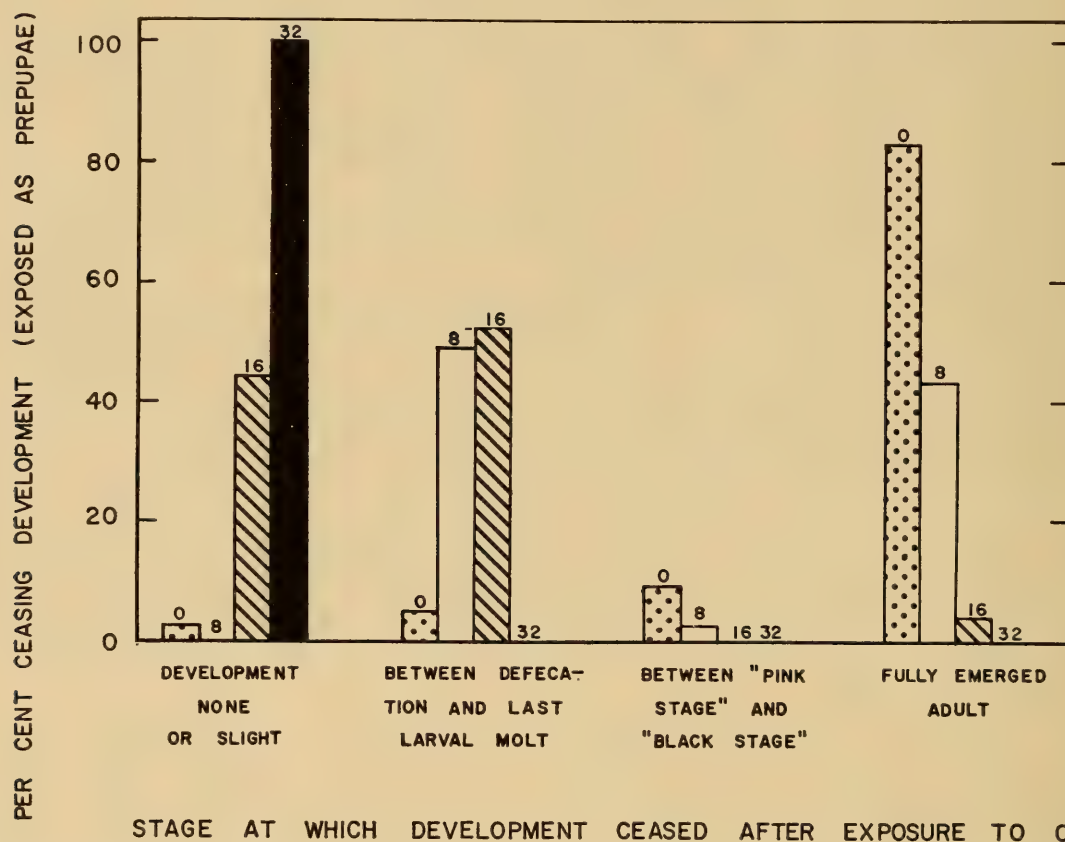


FIGURE 3. The stage of development attained by early prepupae following exposure to 5 atms. oxygen at 30° C. About 50 early prepupae were subjected to each exposure. The duration of exposure is indicated above each bar. Wasps which completed the larval-pupal transformation after exposure to oxygen also successfully completed development and emergence, and almost none of them ceased to develop at stages intermediate between emergence and the larval-pupal transformation. A similar result was also obtained in the experiment with chilled diapausing larvae. For early prepupae, the effect of exposures between 1 and 8 hours duration was not significantly different from an 8-hour exposure.

develop after exposure which markedly reduced adult emergence. As exposure times were increased to 16 hours, fewer animals developed beyond defecation; an exposure somewhere between 16 and 32 hours prevented all further development (Fig. 3). Examination showed that those animals that failed to emerge as adults ceased developing before or soon after defecation. Thus treating larvae and prepupae with oxygen blocked the developmental changes which immediately precede the transformation from larvae to pupae; animals that successfully pupated almost invariably completed adult development and emerged (Fig. 3).

5. The effects of oxygen on "pink stage" developing adults

Approximately 80 per cent of the wasps exposed in the "pink stage" to 5 atms. oxygen for 8 to 16 hours appeared to complete adult development (*i.e.*, reached the "black stage") (Table III). At these doses then, increasing the length of exposure to oxygen had no effect on the normal differentiation and pigmentation of the epidermis and appendages. That the longer exposures in this range were toxic, however, was revealed by a decrease in the ability of the adults to complete emergence. Thus after exposures in the "pink stage" for up to approximately 12 hours, most of the resulting "black stage" wasps emerged; however, after 14 to 16 hours only a fraction of these "black stage" wasps escaped completely from their pupal cuticles (Table III). This is in striking contrast to results obtained with

TABLE III
Development of "pink stage" wasps after exposure to 5 atms. of O₂ at 30° C.

Expt. No.	Exposure (hrs.)	Per cent of wasps exposed*			
		Ceasing development at		Completing adult development**	Completing emergence
		"Pink Stage"	Intermediate stages (between "Pink" and "Black Stage")		
300*	8	15	4	81	77
300	10	14	0	86	65
300	12	19	0	81	19
302*	12	Not determined	Not determined	80	60
301*	14	Not determined	Not determined	85	23
302	14	Not determined	Not determined	82	23
300	16	45	38	17	0
301	16	Not determined	Not determined	73	7
302	16	Not determined	Not determined	79	13
114	24	100	0	0	0

* About 30 individuals used in each exposure in experiment 300; about 25 individuals used in each exposure in experiment 301 and 302.

** "Black stage," partially emerged adults, and adults.

chilled diapausing larvae and prepupae where most animals that failed to emerge also failed to complete adult development (Fig. 3). Exposing "pink stage" wasps to oxygen for longer than 16 hours commonly prevented further development, and the animals remained in the "pink stage" until death (Table III).

The results obtained with the "pink stage" wasps suggest that although exposures up to about 16 hours of oxygen fail to inhibit epidermal differentiation during adult development, some internal system necessary for ecdysis is damaged. Since proper functioning of muscles and nerves is necessary for emergence, and since the development of the muscular system can be easily observed, the effects of oxygen on this system were studied. This proved a fortunate choice.

To study the extent of muscle development, wasps that completed adult development were immersed in Zenker's fixing solution. After 24 hours, the animals were dissected and their thoracic muscles examined. In normal "black stage" wasps and in adults, the thoracic muscles are grouped in 5 longitudinal and 5 dorsosternal pairs. These thick white bands almost fill the thorax and are the most prominent tissue in this part of the animal. Oxygen had a striking effect

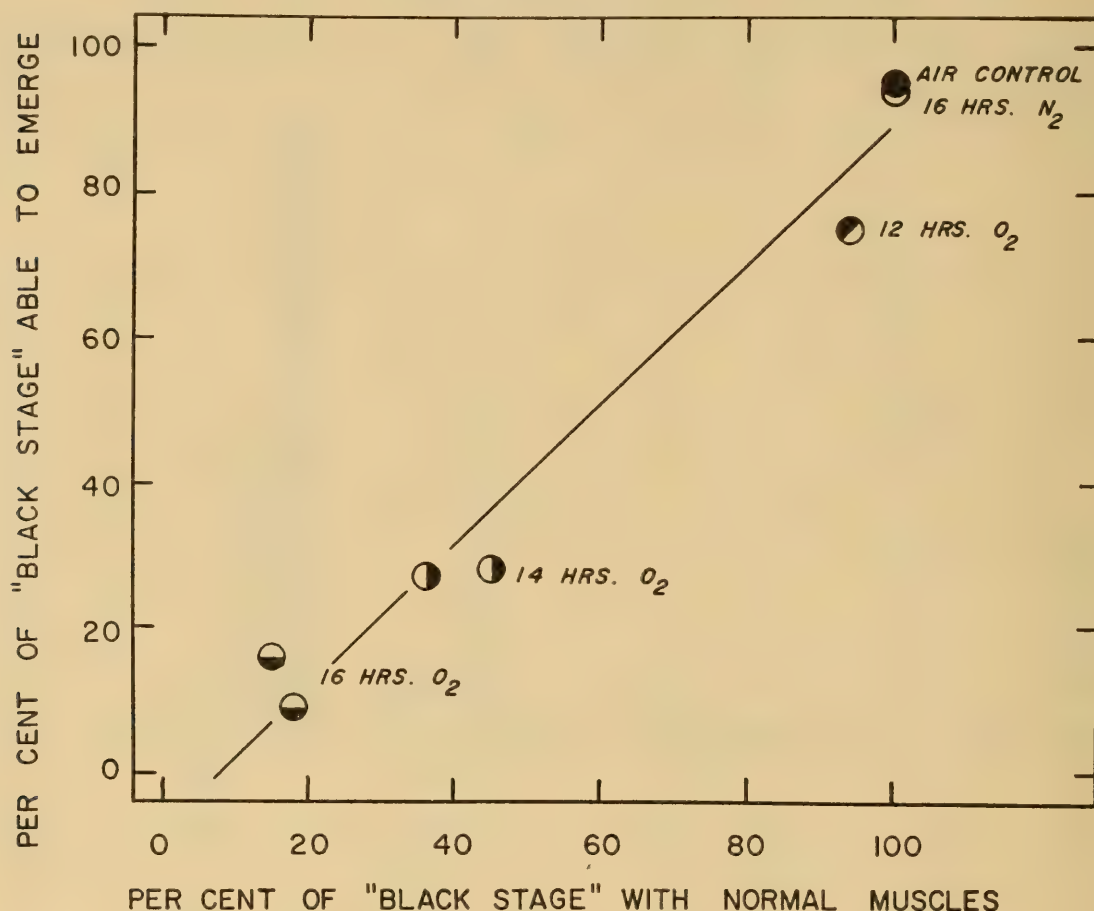


FIGURE 4. The percentage of "black stage" adults which emerge as a function of the percentage which develop normal thoracic muscles. In two experiments (Nos. 301 and 302; see also Table III), "pink stage" wasps of uniform age were exposed to oxygen (5 atms. at 30° C.) for periods that allowed most of them to differentiate to the "black stage." The duration of exposure indicated in the figure varied from a period so brief that almost all adults emerged, to a period so long that no adults emerged. After exposure, the "pink stage" insects were divided into two groups; in one group, the emergence of wasps was observed while in the other group each animal that completed adult development was dissected and its thoracic musculature examined. About 110 insects were dissected.

on the development of these muscles. In "black stage" wasps unable to emerge because of oxygen poisoning in the "pink stage," the thoracic musculature ranged from thin strands of differentiated muscle to a yellowish, jelly-like mass with no distinguishable strands of muscle. The amount of differentiated muscle present was easily recorded as none, small, moderate, or normal.

In the two experiments recorded in Figure 4, it is clear that as oxygen exposure increased from 12 to 16 hours, the decrease in number of adults able to emerge was

paralleled by a decrease in the number of adults that had normal muscles. As previously mentioned, the percentage of animals that completed external adult development after 12, 14, and 16 hours of oxygen was the same (Table III) whereas the percentage of animals that emerged decreased steadily between 12 and 16 hours of oxygen exposure (Fig. 4). After 12 hours of exposure, all of the wasps developed at least a few strands of thoracic muscles. After 16 hours of exposure, however, only 55 per cent of the wasps had any detectable strands (of muscle), and the remainder had only a jelly-like mass in place of the thoracic muscles. Thus, with longer exposures, not only did fewer wasps develop normal thoracic muscles, but the amount of muscle development occurring in animals that failed to emerge also decreased. In our opinion, inability of the oxygen-treated "pink stage" wasps to emerge can be adequately accounted for by the observation that oxygen impaired the development of adult muscles.

6. *The effects of oxygen on "black stage" developing adults*

In most experiments, 2 to 4 hours' exposure to 5 atms. of oxygen at 30° C. caused a detectable decline in the emergence of insects exposed during the "black stage." Several lines of evidence indicate that these wasps were not dead but paralyzed: (a) 24 to 48 hours after exposure of less than 8 hours duration, at the time when they normally emerged as adults, many of the animals moved their appendages weakly; (b) after exposures longer than 8 hours, no movement was observed, but the molting fluid was partially resorbed; (c) animals exposed as long as 8 hours retained a healthy, shiny, black appearance for at least five days, whereas animals killed by ether or alcohol fumes soon became dry, shriveled and dull in appearance. To show that the immobile wasps were still alive, the respiratory rate of several groups of animals was measured before, immediately after, and at intervals of 24 hours following exposure to five atms. of oxygen at 30° C. for various periods. All groups were the same age when oxygen uptake was measured. Since there was no way of knowing how many of the animals in each group were dead or dying as a result of treatment with oxygen, the data summarized in Figure 5 refer to the average oxygen uptake of a group and may not accurately mirror the respiratory behavior of any individual.

The oxygen uptake of the controls was recorded until the adults began to emerge. As seen in Figure 5, the oxygen consumption of the air controls rose sharply on the day of emergence. In the group exposed to oxygen for 4 hours, respiration dropped slightly immediately after treatment, but by the next day had risen to the level of the control. Although adults began to emerge during the next 24-hour period, fewer than 10 per cent completed emergence. Thus, although respiration returned to normal levels, the paralytic symptoms of oxygen poisoning remained. Immediately after 5 hours of exposure, oxygen consumption was only slightly less than after 4 hours of exposure. The initial effects of 8 hours of exposure were more dramatic, and respiration almost ceased. However, 24 hours after exposure to oxygen for 5 or 8 hours, respiration had noticeably increased. This increase in oxygen uptake continued until the second day after exposure. In the case of 5 hours of exposure to oxygen, respiration returned to the pre-treatment level, and yet none of the insects ever emerged. After the second day, respiration gradually declined, probably signifying that the insects

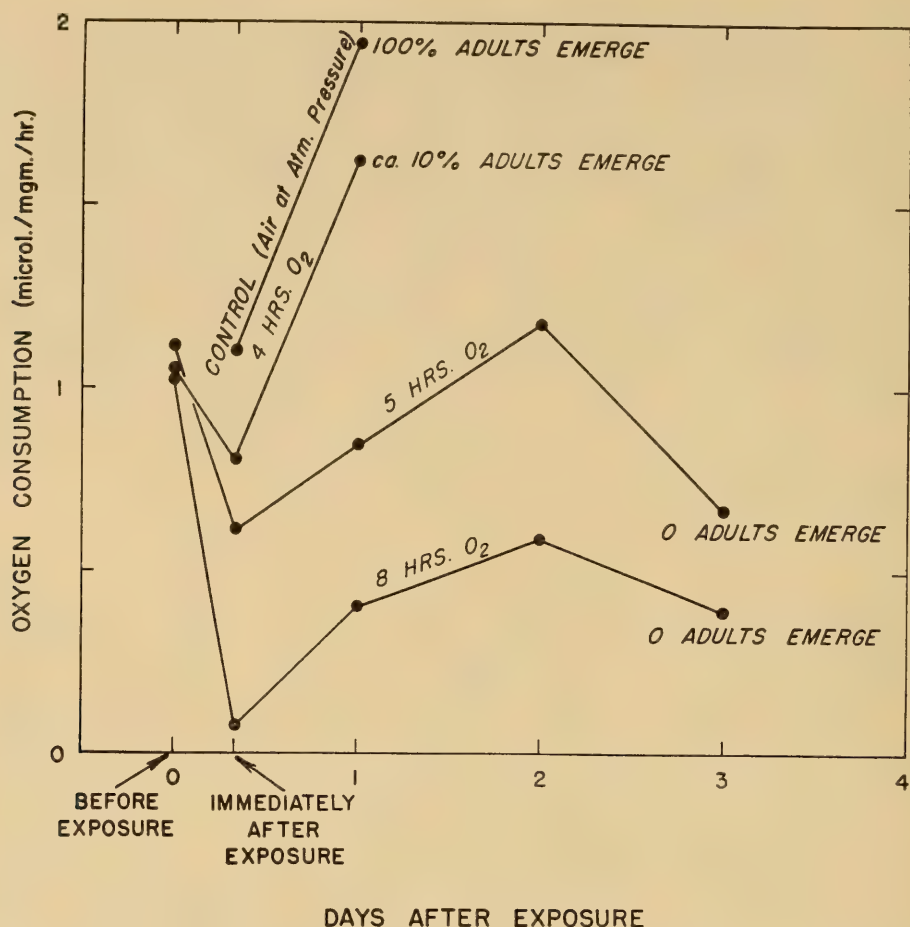


FIGURE 5. The effect of 5 atms. O₂ at 30° C. on the respiration of "black stage" wasps. Groups of 50 wasps were enclosed in perforated gelatin capsules and placed in 20-ml. Warburg vessels, the center wells of which contained 2% KOH. Oxygen uptake was measured immediately before and after exposure of wasps to 0, 4, 5, and 8 hours of O₂. Following this the respiration was measured daily until adults began emerging or until the gradual decline in respiration indicated that poisoned animals were dying.

were dying. Similar results were obtained in each of four separate experiments involving a total of 1100 "black stage" animals. That animals subjected to a short treatment regained a respiration comparable to controls but still failed to emerge, suggests that the system poisoned by threshold oxygen exposure accounts for only a small fraction of the total respiration of the insect.

7. Oxygen poisoning of adults

About 600 adult wasps of known ages were compressed with oxygen to various pressures at 30° C. Males and females alike exhibited successive stages of oxygen poisoning similar to those described by Williams and Beecher (1944) for *Drosophila*:

1. motor activity increased and then gradually declined;
2. walking ceased but a standing position was maintained;
3. the ability to right and to stand disappeared;
4. all spontaneous movements ceased;
5. reflex excitability disappeared.

Since it was difficult to determine the difference between "slight" movement and "no" movement, the time required for loss of the righting reflex was chosen as a convenient measure of oxygen poisoning. In a group of ten adults, the last insect to lose its righting reflex usually did so no more than 5 minutes after the first animal had lost its reflex. Hence it made no appreciable difference in the results whether we measured the exposure time which caused loss of righting reflex in 50 per cent or 100 per cent of the animals.

Figure 6 plots the time for loss of righting reflex as a function of oxygen pressure. In 10 atms. oxygen, adult *Mormoniella* lost their righting reflex after about one-half hour. Poisoning was 8 times faster at 10 atms. than at 3.3 atms. Even at 1.6 atms., the lowest oxygen pressure used in any experiment, poisoning occurred. The relation between pressure and duration of exposure for loss of

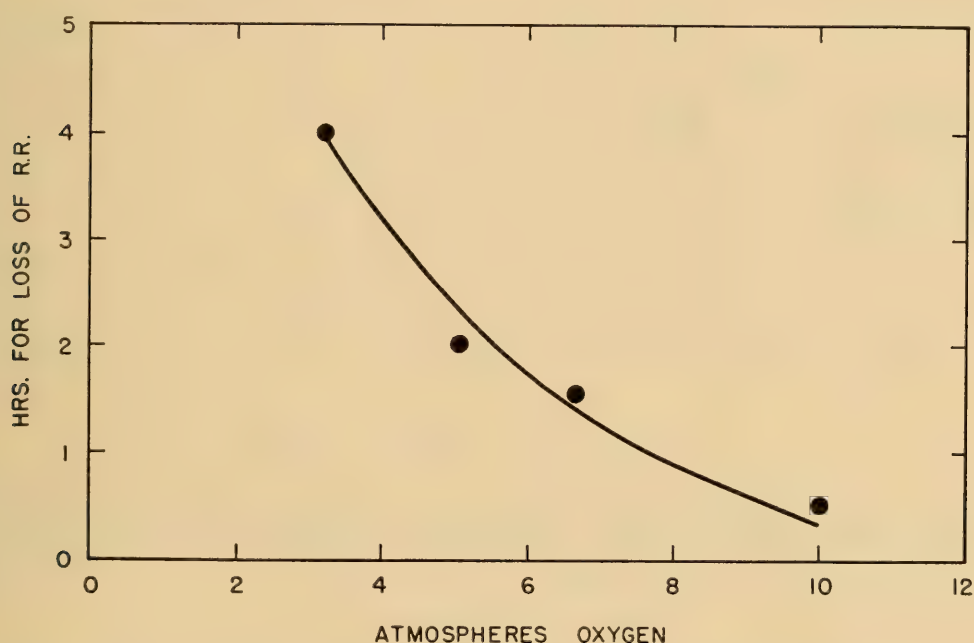


FIGURE 6. Time for loss of righting reflex (R.R.) in 100 per cent of 1- to 2-day-old adult wasps as a function of oxygen tension at 30° C. Twenty adults were exposed at each oxygen tension.

righting reflex appears to be logarithmic, similar to findings of Williams and Beecher (1944) on *Drosophila*.

Both temperature and age affected the sensitivity of adults to oxygen (Fig. 7). Thus the time required for poisoning decreased at higher temperatures; at 10 atms. and 20° C. adults lost their righting reflex in about 60 minutes, whereas at 10 atms. and 30° C. they lost their righting reflex in 20 to 30 minutes. Furthermore, old animals were more sensitive than young ones. Thus 0- to 1-day-old wasps lost their righting reflex in approximately 60 minutes, 1- to 2-day-old wasps in about 40 minutes, and 4- to 5-day-old wasps in about 30 minutes (*cf.* Williams and Beecher, 1944).

Adults removed from oxygen promptly after the loss of righting reflexes generally recovered completely within 24 hours. But animals kept in oxygen for

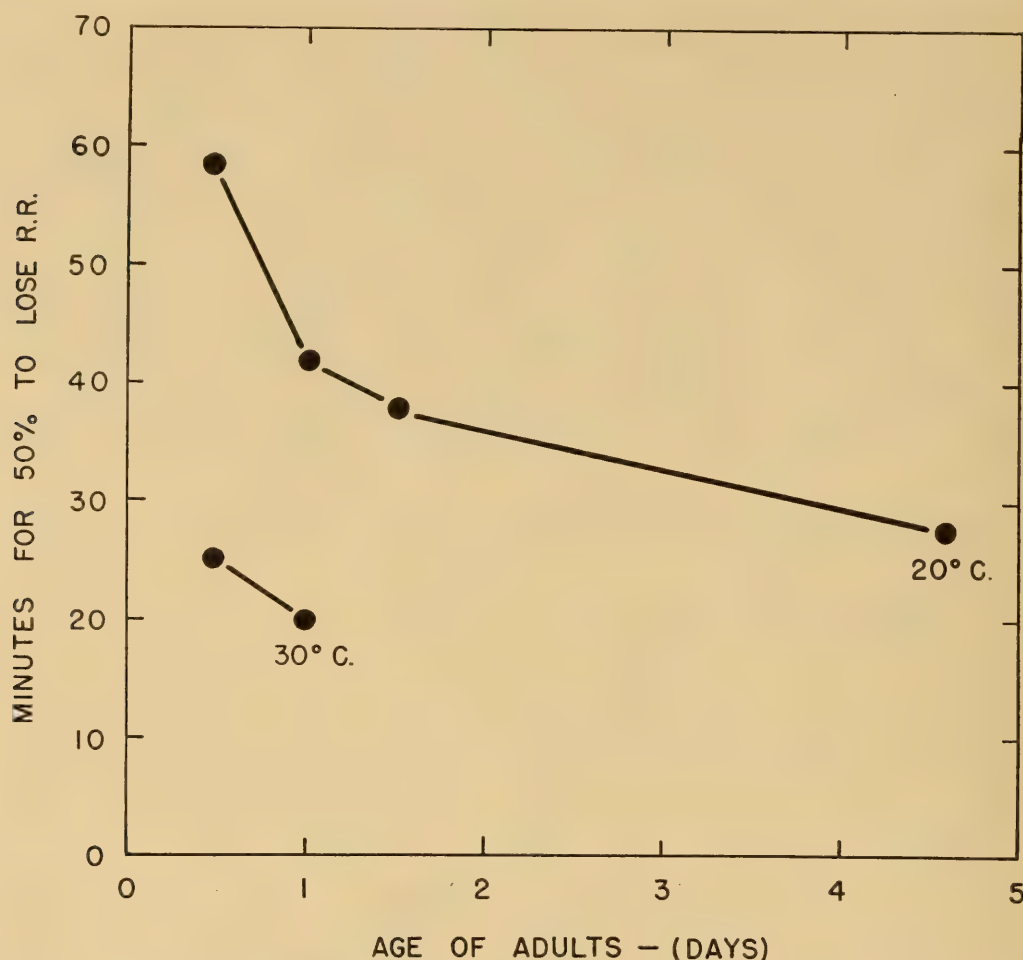


FIGURE 7. Time for loss of righting reflex (R.R.) at 10 atm. O_2 and at two temperatures as a function of age of the adult wasp. Poisoning is more than twice as rapid at 30° C. as at 20° C. At least 50 adults of each age were used at 20° C.; 30 adults of each age were used at 30° C.

as little as 10 minutes after the righting reflex was lost usually recovered only partially and failed to regain normal coordination and motor ability.

DISCUSSION

1. *The sites of oxygen poisoning in Mormonella*

A. *Developmental processes involved*

The present experiments reveal that oxygen can have a variety of toxic effects on *Mormoniella*: it can prevent pupation, it can prevent imaginal muscle development, it can paralyze adults. These different effects on various stages of the life cycle can be understood best by considering the processes occurring at each developmental stage which are likely to be affected by oxygen. We know from ligature experiments on chilled diapausing larvae that at this stage the neurosecretory cells of the insect's brain have not secreted brain hormone and initiated the events leading to pupal development (Malamud, 1958). Thus, for the first few hours after their return to room temperatures, chilled larvae are engaged in

minimal developmental activity, a quiescence which sharply contrasts with the larval-pupal transformation occurring 24 hours later. During the prepupal period the cells of the integumentary imaginal discs divide and spread rapidly, and the discs of the legs, wings and antennae grow out of their sacs. This activity is followed by defecation, whereupon the appendages evert, and a distinct head, thorax and abdomen become visible. Shortly thereafter the last larval cuticle is shed and the antennae, legs and mouth parts promptly attain their full length and adult aspect. During adult development itself the epidermis produces a new cuticle, with ridges, grooves and bristles, which gradually becomes pigmented.

It is noteworthy that as far as external features are concerned, the most marked transformation occurs in the prepupa, not in the developing adult. Furthermore, the prepupal and very early pupal stages are the periods of greatest development of the muscular and nervous systems and of the proliferation of the imaginal discs. Thus, most of the migration and fusion of ganglia in the nerve cord and brain which occurs in the formation of the adult nervous system is completed after the last larval molt and before the "pink stage." Likewise, during the prepupal and early pupal stages, myoblast divisions are completed in the thorax, and presumptive muscle cells begin to fuse. This leads to the appearance, just prior to the "pink stage," of five, thin, syncytial dorsosternal columns of undifferentiated muscle. During the "pink stage" these syncytial columns continue to increase in size, and the cells involved in their insertions differentiate. According to Tiegs (1922), the development of the adult abdominal and leg muscles is similar. Obviously, the normal differentiation of the nervous and muscular systems is necessary if the adult is to emerge.

B. *Effects on larvae and prepupae*

In terms of these developmental processes, it appears that the major difference between the oxygen-sensitive early prepupa and the oxygen-insensitive chilled diapausing larva and "pink stage" (Fig. 2) is the intense proliferation of imaginal discs in the prepupa. In the chilled diapausing larva, this process has not yet begun, and imaginal cells are comparatively inactive; in the "pink stage," proliferation has already been accomplished. Animals with actively dividing cells appear more sensitive to oxygen poisoning than animals in which the division of the imaginal cells has not begun or has just been completed.

C. *Effects on "pink stage" wasps*

The major morphogenetic processes during adult development that determine the ability of the adult to emerge are the differentiation of the muscular and nervous systems. Preventing the differentiation of either muscle or nerve or damaging these systems after differentiation would prevent emergence. The present experiments reveal that oxygen poisoning in the "pink stage" interrupts muscle differentiation and so prevents adult emergence.

The failure of muscle development in "pink stage" wasps exposed to oxygen could be the result of direct damage to the developing muscles. However, it is worth recalling that several investigators have reported that adult muscle development in Lepidoptera is prevented by removal of the pupal nervous system, al-

though development into an otherwise normal adult proceeds (Nüesch, 1952; Williams and Schneiderman, 1952). These observations confirm the original finding of Kopeć (1923) that deganglionation of thoraces of *Lymantria dispar* prepupae prevented the development of thoracic muscles. Kopeć also found that adult development of the integument, as well as the external adult appearance of wings and legs, was not affected by deganglionation. From these results, one might anticipate that if oxygen poisoning severely damaged the nervous system of the "pink stage" *Mormoniella*, muscle differentiation would cease. Whether the failure of muscle differentiation after oxygen poisoning is caused by a direct action on the muscle itself or by an indirect inhibition that disrupts the trophic role of the nervous system remains to be decided.

D. Paralytic effects on "black stage" developing adults and adults

During adult development the neuromuscular system appears to become more sensitive to oxygen poisoning than in the young "pink stage." Thus, when development was allowed to proceed beyond the "pink stage" before oxygen was applied, oxygen sensitivity progressively increased until the "black stage," where even brief exposures prevented emergence. It is of special interest that although these "black stage" wasps were unable to emerge, their respiration recovered and was comparable to the controls. The animals were apparently paralyzed and unable to escape from their pupal cuticles. The observation that this paralysis had no effect on the insect's respiration suggests that either (a) the system first affected by oxygen is responsible for only a small fraction of the total respiration of the animal, or, alternatively, that (b) the respiratory activity of the affected system was not severely damaged. (For interpretation of similar effects in vertebrates see Stadie and Haugaard, 1946.) After exposures to oxygen for 8 hours, the respiration of "black stage" wasps immediately declined, never to return to normal. This permanent decrease in oxygen uptake, evoked by prolonged exposure to oxygen, suggests that the systems of cellular respiration suffered irreparable damage. The inhibition by oxygen poisoning of the respiration of developing insects has also been reported by Clark and his colleagues (Clark and Papa, 1958; and Clark, Harwitz and Rubin, 1958). These workers examined the effects of one minute of exposure to two atmospheres of oxygen on the respiration of the braconid wasp *Habrobracon*. Not unexpectedly, this brief oxygen exposure had no effect on "pigmented *Habrobracon* pupae," which correspond to the "black stage" *Mormoniella*. However, in stages whose subsequent development was adversely affected by these brief oxygen exposures, respiration was immediately inhibited. Clark and his colleagues do not report a recovery of respiration such as we found.

The adult *Mormoniella* was paralyzed by oxygen in much the same way as the "black stage" wasp. The experiments revealed that increasing exposure to oxygen caused an increasing loss of motor activity and coordination. Here again, either the nervous or muscular systems might be the site of oxygen damage.

It seems more likely to us that oxygen acts on the nervous system rather than directly on the muscle. Such a view is consistent with the fact that in oxygen poisoning of vertebrates, when pulmonary damage is avoided, the nervous system is the system first affected and most sensitive to oxygen. Further support for this

opinion stems from the findings, already discussed, that oxygen poisoning of "pink stage" wasps has the same effect on muscle development as deganglionation.

2. Differences in the sensitivity of various organ systems during development

From the foregoing analysis it seems likely that the differences in sensitivity of chilled diapausing larvae, early prepupae, and "pink stage" developing adults (Fig. 2) reflect an increased sensitivity of dividing cells to oxygen. It also seems probable that the gradual increase in sensitivity to oxygen which begins during adult development and continues uninterrupted during adult life reflects an increasing sensitivity of the nervous system to oxygen poisoning. It is interesting to speculate that impulse conduction and transmission are the functions disrupted by threshold oxygen poisoning of "black stage" and adult wasps, while the trophic influence of nerve on muscle is the function impaired in early stages of adult development.

It is noteworthy that exposure to oxygen of "pink stage" wasps may inhibit muscle development, and yet have no effect on the further differentiation of the integument and appendages. This indicates that in the "pink stage" the integument is not as sensitive to oxygen poisoning as is muscle. However, it is important to emphasize that there are times in development when the epidermal differentiation is sensitive to oxygen poisoning. Thus, when early prepupae were exposed to oxygen, many animals failed to develop beyond defecation. This was also true of the less sensitive, chilled diapausing larvae. The larval cuticle was not shed, and the appendages, head, thorax, and abdomen of the adult insect failed to form. We can scarcely attribute the lack of development at this stage to myoblast or nerve cell damage. Failure to develop in the chilled larva and prepupa apparently reflects a failure of the epidermal imaginal discs to fulfill their role.

Differences in the oxygen sensitivity of the various developmental stages of insects have also been reported by Clark and his co-workers (Clark and Herr, 1954). In *Habrobracon* they report that emergence of older prepupae and white pupae was reduced by exposure to one atmosphere of oxygen for one hour at 30° C. This same exposure had no effect on younger larvae-in-cocoons and little or no effect on developing adults. Recently, Clark and Papa (1958) have made the remarkable discovery that exposure to two atmospheres of oxygen for *five seconds* arrested development of 100 per cent of white pupae of *Habrobracon*! From their results it appears that this stage is exceedingly sensitive to oxygen poisoning. In our experiments, higher pressures and much longer exposures (10 atms. for 40 minutes, Table II) were required to inhibit adult development and emergence of *Mormoniella* exposed as early prepupae. (In our experience, the early prepupa is the stage most sensitive to oxygen poisoning.) Although we have not studied the effects of oxygen on *Mormoniella* immediately after the last larval molt, the stage which corresponds to Clark's white pupae, it seems most likely that *Habrobracon* and *Mormoniella* differ considerably in their absolute sensitivity to oxygen poisoning.

In their studies of oxygen poisoning during adult development, Clark and his co-workers did not report a rise in sensitivity during adult development (as reflected in the failure of adults ultimately to emerge), as was found in the case of *Mormoniella*. This is not unexpected, since the exposures they employed were much lower than the ones used in the present experiments.

3. *A comparison of the sensitivity to oxygen poisoning and x-irradiation*

Gerschman *et al.* (1954) suggest that x-irradiation and oxygen poisoning have a common mode of action, both agents causing the formation within cells of large amounts of peroxide and free oxidizing radicals such as OH and OH₂. They support this view with the finding that exposure to high pressures of oxygen after x-irradiation acts synergistically with x-irradiation in decreasing the survival of mice, and that reagents which protect organisms from radiation damage also protect mice from lethal effects of oxygen. The argument gains further support from the fact that oxygen can mimic many of the effects of x-rays, *e.g.* chromosome breakage (Conger and Fairchild, 1952). During the development of *Mormoniella*, however, the sensitivity to x-irradiation (Kuten, 1955; Schneiderman *et al.*, 1956, 1957) does not parallel the sensitivity to oxygen. Although the effects of oxygen poisoning and x-irradiation on *Mormoniella* are strikingly similar during the late larval and pupal stages, during adult development sensitivity to oxygen gradually increases while sensitivity to x-irradiation steadily decreases. In "pink stage" wasps, 4000 r reduced adult emergence by half; in the "black stage" doses as high as 64,000 r failed to inhibit emergence at all (Schneiderman *et al.*, 1956). Similarly, Clark and Mitchell (1951) showed that resistance of *Habrobracon* to x-irradiation increases during adult development. Apparently during adult development systems involved in emergence of the adult wasp become increasingly resistant to x-irradiation while becoming more sensitive to oxygen poisoning. Thus, although x-irradiation and oxygen poisoning may have a similar mode of action on dividing and undifferentiated cells, oxygen has a specific effect on the adult which is not produced by x-irradiation. If oxygen poisoning, like x-irradiation, leads to the formation of free radicals, it is not clear why oxygen has such a toxic effect on adults, while x-irradiation does not.

The type of analysis employed in the present study permits us to define the systems which are the target of oxygen and to recognize that the sensitivity of a developing system can change during its differentiation. Thus although gaps exist in our understanding of the biochemistry of oxygen toxicity, oxygen offers itself as a useful tool to the experimental morphologist (*cf.* Nelsen, 1950; Malamed, 1958). Moreover, for the student of insect growth, the fact that oxygen poisoning appears to provoke many of the same developmental consequences as deganglionation suggests new approaches to investigations of the trophic function of nerve.

We wish to thank Professor Carroll M. Williams for his most helpful comments on the manuscript, and Dr. Timothy H. Goldsmith for his interest in discussing many aspects of this work and care in reading the manuscript. Dr. Williams kindly provided equipment and space for the experiments on muscle development in "pink stage" wasps.

SUMMARY

1. Experiments were conducted to determine the effects of high pressures of oxygen on the development and behavior of an insect, the chalcid wasp, *Mormoniella vitripennis*.

2. More than 10,000 wasps in five different stages of post embryonic development were compressed with 5 and 10 atmospheres of oxygen, nitrogen, or helium. Rapid compression and decompression were detrimental to chilled diapausing larvae but not to other stages. Although the high pressures of helium or nitrogen

had no, or only slight, adverse effect, high pressures of oxygen were exceedingly toxic at certain stages of development.

3. The chilled diapausing larva was but slightly sensitive; about 12 hours at 5 atmospheres oxygen was required to reduce adult emergence by 50 per cent. Sensitivity reached a maximum during the early prepupal stage, when less than one hour of exposure to 5 atmospheres of oxygen reduced adult emergence by 50 per cent. Sensitivity decreased after pupation, and in the "pink stage," at the outset of adult development, about 14 hours of exposure were necessary to reduce adult emergence by 50 per cent. During the rest of adult development and throughout imaginal life, the sensitivity to oxygen again increased. The organ systems attacked by oxygen during these different stages were examined.

4. Exposing "pink stage" wasps to 5 atmospheres of oxygen for about 16 hours stopped development in the "black stage" just prior to adult emergence. Dissection of these "black stage" wasps revealed that they failed to emerge because oxygen poisoning had prevented their thoracic muscles from differentiating. The possibility that this block to muscle development resulted either from direct injury to the muscle cells or from damage to the trophic function of the nervous system was discussed, and an action on the nervous system was favored.

5. Exposure of "black stage" wasps just prior to eclosion caused an immediate and abrupt decline in respiration. Even when respiration returned to control levels within a day, the wasps remained paralyzed within their pupal cuticles and never emerged.

6. The first visible sign of oxygen poisoning in adults was loss of motor activity and coordination. After brief exposures, wasps often recovered completely, but longer exposures evoked permanent paralysis.

7. It was suggested that in the early stages of adult development from the "pink stage" onward, the trophic influence of nerve on muscle is disrupted by oxygen; and that just prior to emergence, and in the adult, impulse conduction and transmission are prevented by oxygen. The gradual increase in oxygen sensitivity during adult development and imaginal life appears to reflect an increasing sensitivity of the nervous system to oxygen poisoning.

8. In contrast to the oxygen-sensitivity of the nervous system, the epidermis of the developing adult was highly resistant to oxygen poisoning. However, prior to the initiation of adult development, epidermal differentiation was blocked by oxygen poisoning. The extreme sensitivity of the early prepupa compared with chilled diapausing larva or the "pink stage" wasp probably reflects the high sensitivity of dividing epidermal cells.

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CIRCULATION IN THE HAGFISH, MYXINE GLUTINOSA L.

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Knowledge of the circulation in fishes and in cyclostomes in particular is very restricted and inadequate for comparison with the data available on circulation in higher vertebrates. *Myxine glutinosa* L., which is the species used for this study, belongs to the class Cyclostomata and the family Myxinidae. These animals represent phylogenetically the most primitive of the existing vertebrates and thus occupy a critical position in comparative interpretations. This study seems to be the first one quantitatively undertaking problems related to the dynamics of circulation in myxinoids.

Fishes are considered to have one circulatory pump, the systemic heart, for the propulsion of blood through both the gill and the general body capillaries. Additional hindrance to circulation exists in the special porta and in some species the kidney capillary systems. The circulatory system in myxinoids is provided with several specialized structures. Apart from the systemic heart in *Myxine glutinosa* there are three accessory hearts on the venous side of the circulation. These are the portal heart, the cardinal hearts and the caudal hearts. The portal heart was first described by A. Retzius in 1822. This heart resembles the atrium of the systemic heart and differs from the other accessory hearts by having a relatively large lumen and striated muscle fibers with a consequent greater pumping capacity. The three orifices of the portal heart, the right anterior cardinal vein, the portal vein and the main portal vein have valves. The two first vessels represent the afferent channels and the latter the efferent channel of the portal heart. The cardinal hearts (Cole, 1926) exist as enlarged anterior extremities of the deep anterior cardinal veins. The caudal hearts (Retzius, 1822) are paired expansions on the caudal veins. The efferent vessels of the caudal hearts unite anteriorly to form the median caudal vein. At this point valves are situated which cause unidirection of the venous blood stream. The present study draws particular attention to the role of the gills in the myxinoid circulation and results are presented which demonstrate an active role of the gills for the propulsion of the arterialized blood.

The six bilateral pairs of gills are located in the region cephalad to the heart and caudal to the big tongue muscle. The gills are connected to the oesophagus through the inner gill ducts which constitute the afferent channels for the respiratory water. After passing through the lense or pouch-like gills, the water flows out through the efferent gill ducts which unite on each side and leave the animal through the two branchial openings. A cross-section through a gill will show tissue that projects inwards from the periphery of the gill as a number of radial

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plates, each one clothed by the walls of two adjacent evaginations differentiated from the epithelial lining of the gill. What may be called gill lamellae are represented by the contiguous walls of two adjacent evaginations plus the intervening vascular tissue.

The afferent gill arteries come from the ventral aorta and form a circular vessel around each efferent gill duct (Fig. 1). From this circle, several radial

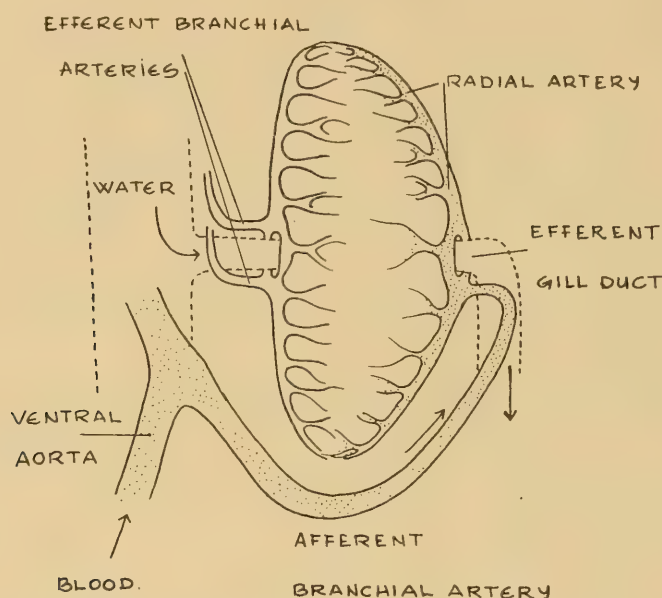


FIGURE 1. (A) Schematic drawing of the vascular arrangement in a gill-body of *Myxine glutinosa*.

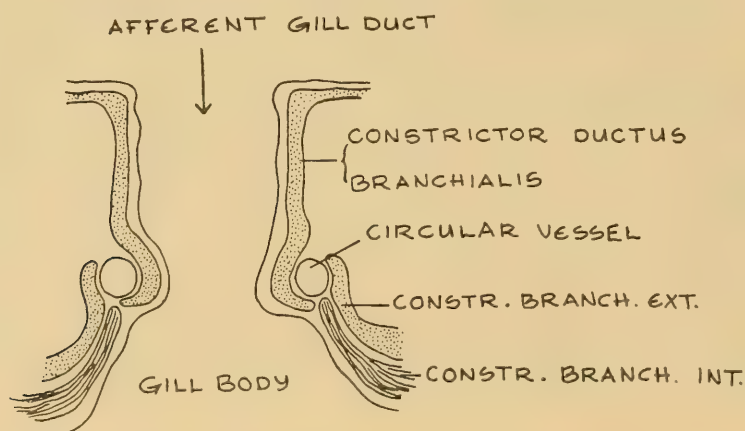


FIGURE 1. (B) Detail showing the topography of a circular vessel and the muscular elements in the gill ducts and gill body.

arteries arise which give off a number of vessels at right angles. These vessels travel in the core of the gill lamellae and join into another series of radial arteries which open into a second circular vessel around the afferent gill duct. From this circular vessel two efferent branchial arteries arise which communicate with the left and right carotid arteries.

A brief histological examination of the gills shows a respiratory epithelium in

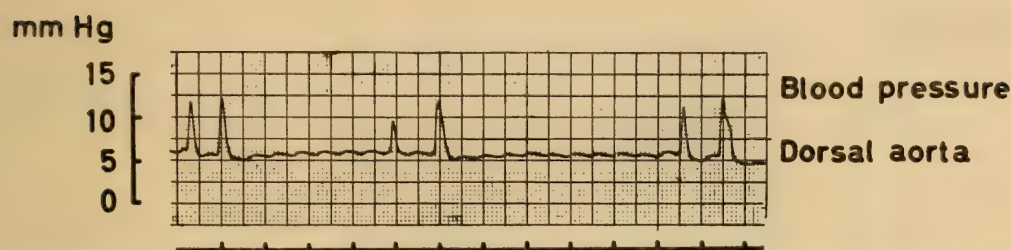


FIGURE 2. Blood pressure record from the dorsal aorta in *Myxine glutinosa*.
Time marks, 4 seconds.

contact with a thin sheet of connective tissue in which the blood vessels are running. Next there are two muscular layers, an external, circular or concentric (constrictor branchialis externus) and an internal radial (constrictor branchialis internus), the axis being the gill ducts. All muscle fibers are distinctly striated. A somewhat similar structure to that in the gill is also present in the gill ducts. The musculature, however, consists only of ring muscles (Fig. 1B). It is of special importance that the gill muscles are not a direct continuation of the gill duct muscles, but that the circular vessels on both sides of a gill body are lying between the ring muscles in the duct and the ring muscles in the gill itself (Fig. 1B). The structure of the myxinoid gills is distinctly different from all other fish-type craniotes, even including petromyzonts.

MATERIALS AND METHODS

The specimens used for this study were trapped near the Dröbak Biological Station in the Oslofjord in Norway. The animals were kept in aquaria supplied with running sea water of 4–7° C. The largest available specimens were used for the experiments. Their length ranged from 25–40 cm. The unanesthetized animal was fastened to a dissection board which was placed in a lucite box filled with oxygenated sea water of 4–7° C. Two incisions were made; the first, in the region 5–8 cm. cephalad to the cloacal opening, exposed the dorsal aorta and the caudal vein. A polyethylene catheter (P.E. 50) was introduced into the dorsal

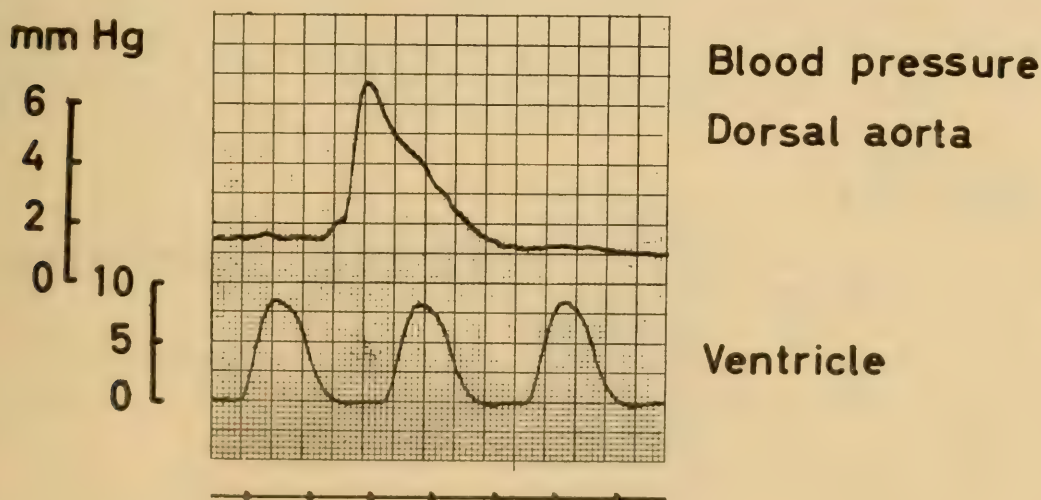


FIGURE 3. Simultaneous blood pressure records from the dorsal aorta (upper curve) and the heart ventricle. Time marks, 1 second.

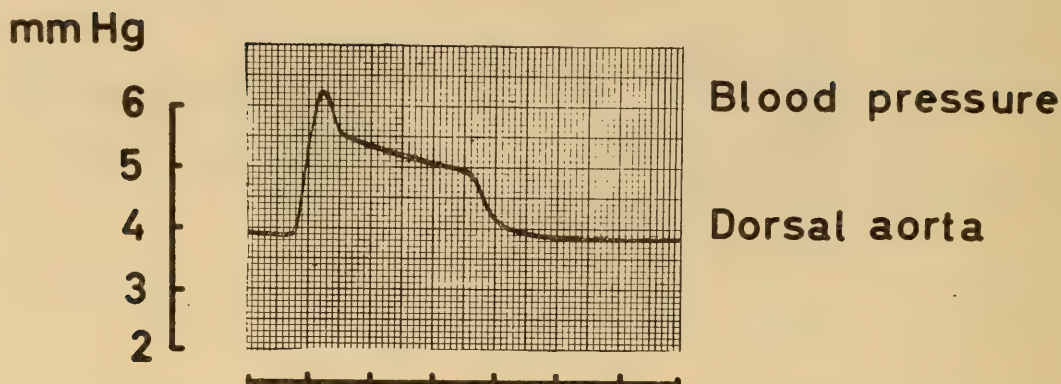


FIGURE 4. Blood pressure record from the dorsal aorta showing sustained gill contraction. Time marks, 2 seconds.

aorta and connected with a Statham or Sanborn pressure transducer. Another incision in the branchial region exposed the systemic and portal hearts. Sometimes the gills themselves were dissected free in order to follow their movements closely. The pressures in the ventricle of the systemic heart and in the portal heart were recorded via cannulas (gauge 26) to the pressure transducers. Care was taken not to alter the horizontal position of the animals during the experiments and temperature changes in the lucite box were avoided. All recordings were made on a Sanborn 4-channel recorder.

RESULTS

The systemic heart showed a frequency different from that of the portal heart. Both the systemic and portal hearts increased their frequency as a result of increased venous return brought about by digital manipulation of the great veins.

The blood pressure recorded in the dorsal aorta showed a pattern like that demonstrated in Figure 2. The smaller oscillations are transmissions of the systemic heart beat through the gill capillaries. The larger paired pressure pulses were the result of active contractions of the gill musculature. These contractions most often came in pairs separated by variable times. The frequency of the gill contractions varied greatly, with average values of 8–10 per minute. Likewise, the absolute values of blood pressures in the dorsal aorta, as well as the rate of the systemic heart, showed great individual variations. The heart rates showed

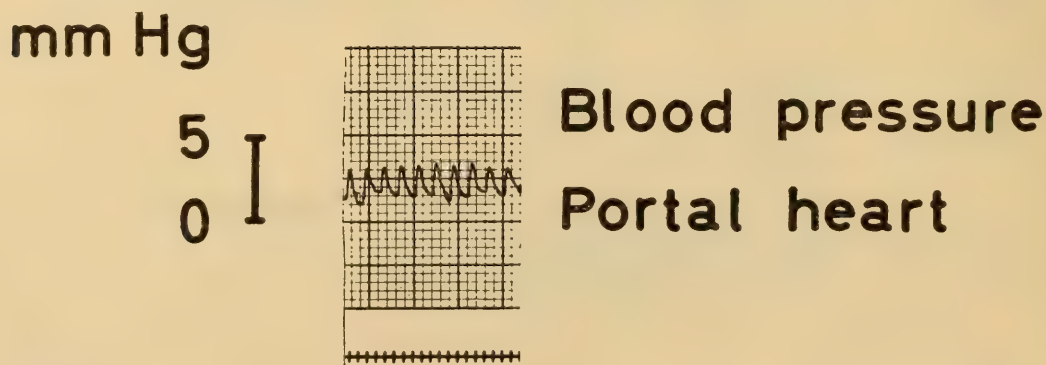


FIGURE 5. Blood pressure record from the portal heart. Time marks, 1 second.

variations between 30–40 beats per minute. The mean blood pressure in the dorsal aorta most often showed values around 5 mm. Hg, varying between 3–8 mm. Hg. The pulse pressures resulting from the systemic heart beat showed values of around 2 mm. Hg or 25 mm. of water. All these pressure values were recorded in the caudal part of the animal in the dorsal aorta. Figure 3 demonstrates pressures in the heart ventricle and dorsal aorta recorded simultaneously. The blood-pressure drop across the gill capillaries calculated from the average pressures in the heart ventricle and dorsal aorta amounted to 4.0 mm. Hg. The lower tracing in Figure 3 shows that intra-ventricular systolic pressure is around 8.5 mm. Hg while the diastolic pressure is zero. During other experiments the same values varied between 15 mm. Hg and zero. Figure 4 shows another feature of the gill contractions, namely the ability to sustain the contractile state for some seconds. The pressure in the portal heart (Fig. 5) was consistently between 3–6 mm. Hg.

DISCUSSION

The circulation in *Myxine glutinosa* as demonstrated in this study differs markedly from circulation in all other vertebrates studied. Most surprising is the active role of the respiratory organs for the propulsion of blood. The unique morphology of the gills in *Myxine*, however, with the striated musculature and special vascular architecture might give reasons for anticipating an active participation of the gills in the circulation of blood. In fact, this is what M. Hofbauer (1937) suggests in her excellent description of the histology of the gills in *Myxine glutinosa*.

It is apparent from Figures 2 and 3 that the pressure wave resulting from a gill contraction has a very steep diastolic gradient. This means that the peripheral resistance is low. In attempting to elucidate the physiological importance of the gill contractions, one has to bring a disputed problem into consideration. This is the problem of lacunar circulation in vertebrates, first described by Rathke in 1825. Cole (1926) pointed out that some of the lacunar spaces (blood sinuses) in *Myxine* contain red blood cells and he called them "red lymphatics." These cannot be considered true lymphatics but must be considered a part of the blood vascular system. The circulatory system of *Myxine* must thus be considered a semi-closed system where the true blood vessels at some particular sites are interrupted by lacunar spaces. Blood enters these spaces directly from the arteries through vascular papillae on the branchial and carotid vessels among others. The communication between the lacunar spaces and the veins are established with valved apertures (see, also, Marinelli, 1956).

A circulatory system of this type can evidently not work as a high pressure system. On the basis of this knowledge the gill contractions with the consequent steep transient pressure rises are well explained. Thus it can be observed through the vessel wall of the dorsal aorta that a steady, slow forward movement of blood takes place, interrupted at each gill contraction by a massive forward displacement. The steady, slow flow component apparently results from the permanent pressure gradient along the arteries between the pressure head from the systemic heart beat, and the veins. The described ability of the gills in *Myxine* to sustain the contractile state for some time seems to be of physiological importance to the animal by increasing the volume of blood displaced during a gill contraction. A con-

tinuous, repeated blood transport of this type obviously necessitates the mentioned properly sited valves and accessory "hearts" on the venous side of the circulation.

The events in the gill pouch during contraction of the gill musculature are probably as follows: The two circular vessels on the afferent and efferent branchial arteries, at the entrance and exit of each gill, will each be pressed together when the striated musculature in the gills and gill ducts contracts. By this act the blood already arterialized will be forced out in the arterial trunks through the efferent branchial arteries, thereby causing the relatively high transient pressure rise throughout the arterial system. On the afferent or venous side of the gill the inflow of venous blood will stop during gill contraction. At the same time the water in the gill pouch has been pressed out, which means that the gill when contracted is in a state where the respiratory function is temporarily stopped. In other words the gills at this time are solely engaged in the circulation of blood and water. Upon relaxation of the gill muscles the inflow of water and venous blood again takes place and the gills resume their respiratory function.

Considerable interest relates to the events in the ventral aorta during a gill contraction. Recording of the pressure in this vessel without interfering with the normal circulation is extremely difficult and has not yet been successful. It may, however, be suggested that a simultaneous contraction of the gills and the systemic heart creates a pressure in the ventral aorta which surpasses a threshold necessary to fill the lacunar spaces through the vascular papillae on the afferent branchial vessels. It may well be reasoned that the outflow of blood through these papillae and into the lacunar spaces has a pressure threshold only brought about by a high pressure in the ventral aorta resulting from a simultaneous contraction of the gills and the systemic heart.

A comparison of the absolute values of pressure in the vessels and heart of Myxine with those reported earlier on selachians and teleosts are probably of limited value, considering that lacunar circulation is not present in the latter. The pressure drop across the gills, although these too differ markedly in their anatomy from gills in selachians and teleosts, is likely of some interest. Thus, Schoenlein and Willem (1894) working with selachians reported a pressure drop in per cent of values proximal to the gills of from 35 to 65%. They worked with *Torpedo* sp. and *Scyliorynchus catabus*. Lyon (1926), working with *Carcharias* sp., reported around 25% pressure drop, while J. Mott (1951) recorded 40 to 50% pressure drop in the common eel, *Anguilla anguilla*. The present study gave values of around 50% pressure loss across the gills measuring pressures in the ventral aorta and heart ventricle. During these recordings the catheter in the dorsal aorta was pushed in cephalad direction to avoid the pressure drop along the dorsal aorta itself. The size of the catheter, however, did not obstruct the circulation in the posterior direction in this vessel.

Another feature of probable physiological importance is explicitly demonstrated in the architecture of the myxinoid gill (Fig. 1). This has to do with the principle of counter currents, recently frequently stressed as being of great importance for efficient exchange of materials and energy as well as for maintenance of concentration gradients in biological systems (Scholander, 1958).

In conclusion it may be stated that the circulatory system in myxinoids, as judged from this study, shows a very decentralized mode of functioning. In other

words the components actively taking part in the circulation of blood are distributed throughout the circulatory pattern. Hence the situation differs greatly from the circulation in other vertebrates where the systemic heart is considered the predominant site of the blood-moving power.

SUMMARY

1. Electromanometers have been used to study the pressure relations in the vascular system of *Myxine glutinosa* L.

2. The data demonstrate a low arterial pressure level in this species correlating with the existence of a lacunar circulation.

3. The average pressure in the dorsal aorta ranged from 3–8 mm. Hg with a pulse pressure of around 25 mm. H₂O. The average pressure drop across the gills, calculated from the average pressures in the heart ventricle and dorsal aorta, was found to be around 4 mm. Hg.

4. The gills have been shown to take active part in the forward propulsion of arterialized blood. This is accomplished by contraction of striated muscular elements in the gills and gill ducts.

5. It is concluded that the circulation in *Myxine* differs from circulation in vertebrates generally by a partial shifting of the blood-moving power from the systemic heart to the gills and the portal, cardinal and caudal auxiliary hearts.

I am indebted to Professor Dr. Björn Föyn and cand. real. Finn Walvig at the Dröbak Biological Station for providing the material used in this study.

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THE EXCRETION OF INULIN AND GLUCOSE BY THE CRAYFISH ANTENNAL GLAND¹

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The anatomy of the crayfish³ green gland (kidney) is relatively well known, due principally to the investigations of Marchal (1892), Peters (1935) and Maluf (1939). It consists, proximal to distal, of a coelomosac (saccule), a sponge-like labyrinth, an elongate (*ca.* 3 cm.; Maluf, 1941a) tubule and a bladder. The bladder empties to the exterior of the crayfish by a short duct.

Several investigations of the physiology of the green gland have been undertaken, but little in the way of unequivocal evidence has been gained concerning its functional mechanism. Schlieper and Herrmann (1930) were the first to discover that crayfish urine is markedly hypotonic to the blood. Peters (1935) removed urine samples from various parts of the green gland. He found that the chloride content of the samples removed from the distal portions (distal tubule and bladder) was much less than the chloride content of urine samples removed from the proximal portions (coelomosac and labyrinth). The chloride content of urine samples removed from the coelomosac and labyrinth approximated that of the blood. Peters concluded from his experiments and observations of the microscopic anatomy of the crayfish green gland that the primary urine, in the form of a blood filtrate, was formed in the coelomosac and modified by reabsorption of salts (and possibly water) in the parts distal to the coelomosac. Picken (1936) made measurements of the colloid osmotic pressure of the blood and the hydrostatic pressure within the sternal sinus of the crayfish. Since the former pressure (average = 15 cm. H₂O) was exceeded by the latter pressure (average = 20 cm. H₂O), Picken concluded that filtration within the crayfish kidney was possible. Maluf (1941b) analyzed the manner in which the crayfish (*Procambarus clarki*) green gland excreted inulin, xylose and various dyes. Since such substances as inulin and xylose are not excreted by the vertebrate aglomerular (secretory) kidney, Maluf reasoned that the crayfish kidney would or would not excrete those substances depending upon whether that organ formed the primary urine by secretion or filtration. Despite the fact that both inulin and xylose were excreted by his experimental animals, he chose to interpret his data to indicate that the

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² Public Health Service Post-Doctoral Research Fellow.

³ Until a recent revision by Bott (1950), the systematics of European crayfishes has been greatly confused. The species name, *Potamobius fluviatilis* used by Marchal (1892), Peters (1935), Schlieper and Herrmann (1930) and Picken (1936) undoubtedly represents at least two species. *Potamobius fluviatilis* used by the northern European workers, probably refers to the present-day species *Astacus astacus* (L.), while that name used by French and British workers probably refers to the present-day species, *Austropotamobius pallipes pallipes* (Lereboullet).

crayfish green gland forms urine by active secretion of all components, including water. Martin (1957) suggested that Maluf's data could alternatively be interpreted to indicate that filtration occurs in the crayfish green gland.

In the present paper, evidence will be presented which will attempt to explain Maluf's inulin and xylose (glucose) excretion data within the framework of a filtration hypothesis.

METHODS AND MATERIALS

The crayfishes used in this study were specimens of *Procambarus clarki* (Girard), *Orconectes virilis* (Hagen) and *Pacifastacus leniusculus* (Dana). No species differences were noted in any of the experiments herein described. The experimental animals were kept in a water bath at 16° C., and all experiments were carried out at that temperature.

Blood for inulin and glucose analyses was collected as follows: About 0.06 cubic centimeter of blood was drawn into a one cubic centimeter tuberculin syringe fitted with a 26 or 27 gauge needle after puncturing the ventral membrane between the merus and carpus of the cheliped (claw). The blood was deposited onto a piece of Parafilm, rapidly drawn into a 50-microliter self-leveling pipette, and transferred to a 12 cubic centimeter centrifuge tube. The protein was precipitated by Flemister's (1958) modification of the Somogyi (1930) method. The precipitate was shaken briefly and centrifuged at 3500 revolutions per minute for five minutes. The supernatant fluid was poured into a ten cubic centimeter test tube. The precipitate was then broken up with a glass rod, washed with a one cubic centimeter aliquot of distilled water, centrifuged, and the solution was added to the first supernatant. The chemical analysis for inulin was done using Flemister's (1958) modification of the anthrone method introduced by Young and Raisz (1952). Glucose was analyzed by the glucose oxidase (GLUCOSTAT, Worthington Biochemical Corp., Freehold, N. J.) method of Saifer and Gerstenfeld (1958).

Urine for inulin, glucose and sodium analyses was collected as follows: The front openings of the crayfish's gill chambers were plugged with a cotton wad and the epistomal area blotted with absorbant paper. The crayfish was held ventral side up under a dissecting microscope (observed at *ca.* 15×) and the urine collected from the nephropore by gently tapping the operculum with a capillary (see Maluf, 1941a, for details of the anatomy of the crayfish nephropore). The capillaries had been drawn previously to a blunt tip and fire-polished. This method of urine collection obviated any danger either of contaminating the urine with blood (as possibly done by Picken, 1936, and others) or making corrections for water loss when the urine is collected by suction (as necessitated by the method of Maluf, 1941b). Urine for chemical determinations of inulin and for glucose analyses was treated in the same manner as described above for blood. Urine for sodium analysis was directly diluted to an appropriate volume with distilled water.

Blood and urine samples for inulin-C¹⁴ analysis were collected volumetrically as described above, but the samples were plated directly onto one-inch planchettes, diluted with two cubic centimeters aliquots of distilled water, and dried. The samples were counted with a conventional windowless gas-flow counter.

Sodium analyses were made with a Coleman flame photometer. Non-radio-

active inulin was analyzed with the aid of a Bausch and Lomb, "Spectronic 20" colorimeter.

The general experimental procedure was as follows: crayfishes were removed from the water bath, wrapped in a paper towel, and permitted to drain for one-half hour. They were then weighed and the urinary bladders emptied. Fifty-microliter urine samples were saved for sodium, inulin, and/or glucose analyses, and the remainder of the urine discarded. Initial blood samples were taken and any material to be injected was placed into the sternal sinus with the aid of a $\frac{1}{4}$ -cubic centimeter tuberculin syringe fitted with a 26 or 27 gauge needle. The inulin, glucose and phlorizin concentrations were adjusted so that volumes no larger than 0.1 cubic centimeter were injected into the animals. The crayfishes were marked on the branchiostegum (gill chamber) with a grease pencil as a means of

TABLE I
Changes in sodium content of urine taken from experimental animals injected with inulin (I) and the control (C)

Animal number	Time (hours)	Urine Na (% orig.)	Time (hours)	Urine Na (% orig.)	Time (hours)	Urine Na (% orig.)	Time (hours)	Urine Na (% orig.)
I-1	2	*	9	*	22	283	59	341
I-3	8	470	22	450	34	875	57	960
I-5	2	*	7	*	32	*	55	75.0
I-6	2	*	7	*	19	*	34	411
I-8	3	*	9	*	21	258	**	**
I-9	3	56.0	9	40.2	21	80.0	**	**
I-10	3	133	9	*	21	57.4	**	**
I-11	3	*	9	75.0	21	89.7	**	**
I-14	3	127	9	141	21	246	30	Died
C-2	2	62.2	9	138	22	214	34	411

* No samples obtainable.

** No sample taken.

identification and returned to the water bath. At definite intervals thereafter, the animals were removed and urine and blood samples taken.

Controls were run with all experiments. The control crayfishes were those into which no initial injection of inulin, glucose or phlorizin was made, but blood and urine samples were taken as described above.

All crayfishes were assigned a reference letter corresponding to the particular substance which was injected into them (*e.g.*, G for glucose, P for phlorizin). This reference letter is reflected in the tables. Further, a particular number and reference letter was assigned to only one animal. Thus, animal G-6 in Table II is the same as animal G-6 in Table III.

RESULTS

Inulin

Of a total of 18 animals injected with inulin, only six continued to produce urine throughout a period of six to 57 hours (see Fig. 1). Five of the remaining 12 began to produce urine within 21 to 55 hours after injection. In the latter

TABLE II
Blood and urine glucose levels (in mg. %) in experimental animals (G) and controls (C) after the injection of glucose

Animal no.	Period 1			Period 2			Period 3			Period 4			Period 5		
	Time (hours)	Blood glucose	Urine glucose	Time (hours)	Blood glucose	Urine glucose	Time (hours)	Blood glucose	Urine glucose	Time (hours)	Blood glucose	Urine glucose	Time (hours)	Blood glucose	Urine glucose
G-1	0	0	0	1	11	0	4	2	0	7	0	0	23	0	0
G-6	0	5	0	1	65	*	4	83	0	7	90	*	58	57	10
G-7	0	5	0	1	93	0	4	80	0	7	62	*	58	19	3
G-11	0	*	0	1	192	0	4	56	7	7	18	0	50	17	0
G-15	0	24	5	1	100	0	4	7	0	7	28	0	54	4	0
G-21	0	10	0	1	560	0	4	220	30	20	10	0	48	10	0
G-22	0	0	0	1	210	0	4	50	0	20	0	0	**	**	**
C-4	0	5	0	1	*	0	4	*	0	7	100	*	19	52	*
C-5	0	28	0	1	45	*	4	56	*	7	*	*	58	57	10
C-12	0	10	0	1	26	0	4	38	0	7	*	*	48	20	*
C-20	0	91	0	2	89	*	25	88	*	48	77	6	**	**	**

* No sample obtainable.
** No sample taken.

TABLE III
Changes in sodium content of urine taken from experimental animals injected with glucose (G) and controls (C)

Animal number	Time (hours)	Urine Na (% orig.)	Time (hours)	Urine Na (% orig.)	Time (hours)	Urine Na (% orig.)	Time (hours)	Urine Na (% orig.)
G-1	1	167	4	200	7	400	23	334
G-3	1	*	4	*	7	*	22	714
G-6	1	*	4	120	30	*	50	82.6
G-7	1	45.5	4	45.5	30	75.0	58	45.5
G-9	1	1125	4	*	23	1850	50	364
G-10	1	13.9	4	*	22	*	50	18.4
G-11	1	100	7	3401	22	66.7	50	167
G-13	4	637	7	341	26	291	54	1045
G-14	4	31.0	7	116	26	35.7	54	*
G-15	1	120	4	124	26	112	54	84.5
C-4	1	150	4	100	7	*	19	*
C-5	4	108	7	95.8	30	120	58	275
C-12	1	100	4	100	20	408	48	125

* No sample obtainable.

cases, the urine/blood (hereafter designated as U/B) inulin values (in milligrams per cent) were 160/0, 60/0, 280/12, 800/440 and 790/498. Of the seven animals that did not produce urine after the injection of inulin, three died. Autopsy of the three crayfishes revealed collapsed urinary bladders. The remaining four

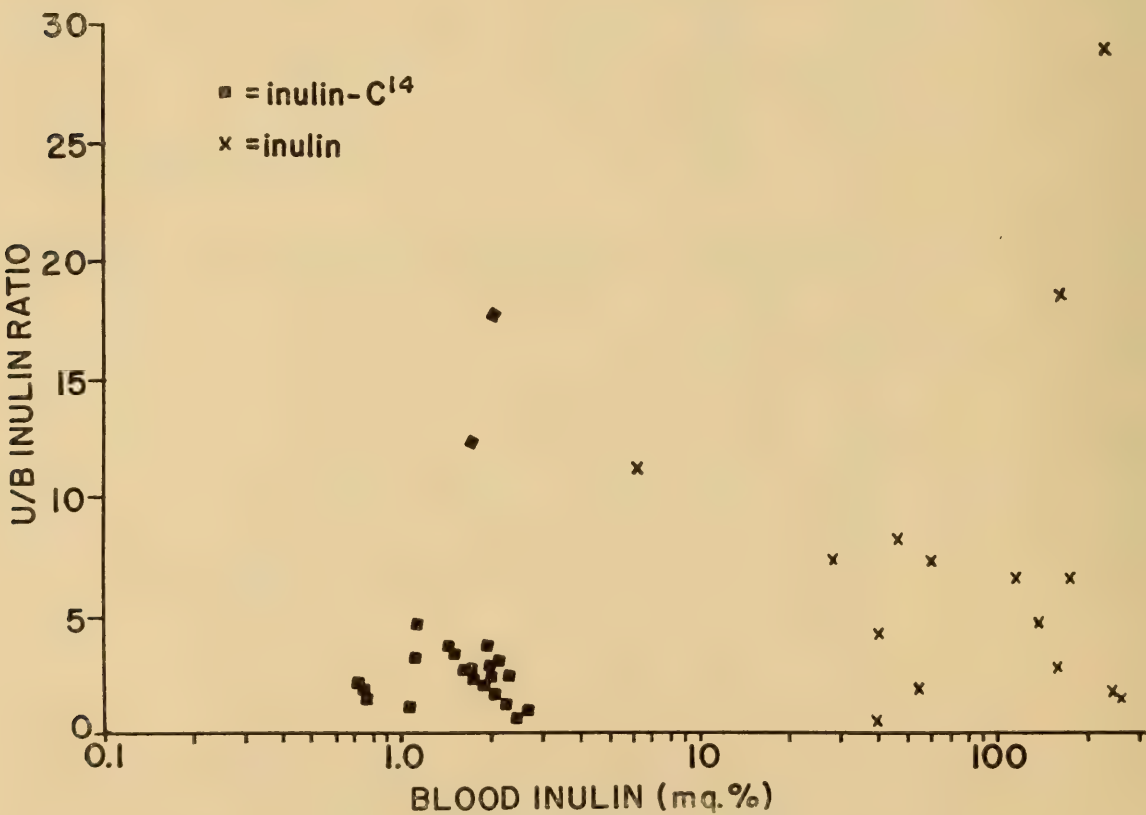


FIGURE 1. Urine/blood (U/B) inulin ratios in relation to blood inulin levels in crayfishes injected with radio-active and non-radio-active inulin.

crayfishes may have begun again to produce urine, but no attempt was made to follow urine production longer than 60 hours.

Eleven animals were injected with inulin- C^{14} , and eight of them continued to produce urine throughout the period of the experiments (six hours; see Fig. 1).

It is interesting to note that whenever only a small amount of urine (less than 10 microliters) could be collected, the inulin U/B ratios were significantly higher than when amounts greater than ten microliters could be collected. Of a total of 39 urine collections during these experiments, 27 were greater than ten microliters (average = 41.5 microliters). The average U/B inulin ratio at the same times was 3.4. The average amount of urine collected during the 12 remaining periods was 5.8 microliters, during which times the average U/B inulin ratio was 9.0. This difference between average inulin U/B ratios of large and small urine samples is statistically significant at the 5% level ($t = 2.25$). Sodium determinations were made on urine collected from nine animals into which inulin had been injected. Urine sodium increased by factors of 1.3 to 9.6 in six of the nine crayfishes (Table I). The sodium content of the urine from two of the three remaining crayfishes dropped after the inulin injection and then rose slightly during the balance of the experiment, although never attaining the concentration of the initial urine sample. The ninth animal stopped producing urine after the injection of inulin and 55 hours later the urine sodium was only $\frac{3}{4}$ that of the initial urine sample. The average sodium content of seven urine samples of less than ten microliters (average = $3.5 \mu\text{L}$) was greater by a factor of 5.1 than the average sodium content of 12 urine samples greater than ten microliters (average = $35.4 \mu\text{L}$). This result is statistically significant at the 1% level of probability ($t = 3.31$).

Glucose

A total of fourteen crayfishes were injected with varying amounts of glucose. Of that number, one-half produced urine relatively continuously throughout the experimental period. The results of analyses of blood and urine glucose from these animals are shown in Table II, where it can be seen that glucose usually (but not always) appears in the urine under one of two circumstances: (1) the blood glucose level exceeds approximately 200 milligrams per cent (animals 11 and 21 at four hours); (2) the experiment lasts thirty hours or longer. Of seven animals that stopped producing urine after the glucose injection, two died, and urine was unobtainable from two others during a period of 21 to 50 hours; the remaining three again produced urine 23 to 26 hours after injection, and in all cases, appreciable quantities of glucose were present in the urine. Of special interest is the fact that analysis of urine and blood of the controls (crayfishes not injected with glucose; see Table II) produced results similar to those obtained from the experimental animals. That is, blood glucose rose, urine production stopped, and in one case, glucose appeared in the urine late in the experimental period.

Analysis of the urine sodium content of ten crayfishes into which glucose had been injected showed that the sodium content of the urine of eight animals increased by factors of 1.2 to 34 (Table III). Two animals, unexplicably, showed a reduction in the sodium content of their urine.

Phlorizin caused the appearance of glucose in the urine in all crayfishes into which it was injected. However, in order to cause the appearance of quantities of

TABLE IV

Blood and urine glucose levels (in mg. %) in experimental animals injected with phlorizin plus glucose (P) and the control

Animal no.	Time (hours)	Blood glucose	Urine glucose	Time (hours)	Blood glucose	Urine glucose	Time (hours)	Blood glucose	Urine glucose	Time (hours)	Blood glucose	Urine glucose	Dosage levels
P-1	0	42	*	4	31	16	10	39	5	24	40	3	12 mg./Kg.
P-2	0	27	0	4	36	65	10	33	14	23	38	6	102 mg./Kg.
P-3	0	39	0	4	51	10	10	44	31	23	32	46	72 mg./Kg.
P-5	0	22	0	4	36	43	10	17	48	23	27	18	32 mg./Kg.
P-6	0	23	0	4	47	106	10	28	59	26	25	27	77 mg./Kg.
P-7	0	31	0	4	70	247	10	36	119	26	58	93	53 mg./Kg.
C-1	0	35	0	4	44	0	10	26	0	**	**	**	**

* No sample obtainable.

** Animal killed accidentally.

glucose sufficient to provide unequivocal results from the glucose analyses, priming with exogenous glucose was necessary. The data shown in Table IV are taken from six animals into which doses of 12 to 102 milligrams per kilogram of phlorizin (plus glucose) were injected. The effect of all tested levels of phlorizin was immediate and prolonged. Differences in effect between the different phlorizin dosage levels appeared to be very slight, although no attempt was made to determine minimal and maximal doses.

DISCUSSION

It would be well at the outset to attempt to clarify the terminology to be used lest the application here of terms coined for vertebrate function lead to misunderstanding. By "secretion" is meant the movement of material *from* the blood *into* the lumen of the organ against a gradient of electrochemical potential. In secretion the cells are expending energy to move solutes and/or water into the lumen. The term "filtration" usually is used quite specifically to indicate the bulk movement of a solution through a porous membrane by the application of a head of pressure. The pore dimensions of the membrane determine what is filtered and what is restrained. Apart from the fact that the necessary net pressure may exist in the crayfish (Picken, 1936), there is no evidence that filtration in this sense occurs among Crustacea. In the following discussion, filtration will be used to mean the formation somewhere in the organ of a (blood-isotonic) solution containing molecules below a critical size. The process is indiscriminate in the sense that the chemical species is not important (*i.e.*, everything below a certain size appears in the "filtrate"). In addition, and critical from the point of view of active water movement, movement of solution occurs from high to low potential. Thus, the question that is really under exploration is, does the movement of water into the lumen of the antennal gland require energy? The formation of a dilute urine by a secretory mechanism would obviously require active water transport.

Factors affecting the variability in urine production

The results of the experiments presented in this paper are similar in most respects to those of Maluf (1941b), except that Maluf apparently did not encounter

difficulty in collecting urine after the injection of inulin and glucose (xylose). A difference in experimental procedure between the present work and that of Maluf's may clarify this discrepancy. Maluf made no effort to obtain urine from his experimental animals for eight to fifteen hours after the injection of inulin or glucose (xylose). At that time, he sacrificed the crayfishes and collected the urine in the bladders. In the present work, attempts were made to collect serial samples at much shorter intervals after the injection of inulin and glucose.

The cause of the aforementioned variability in urine production is unknown. However, the only factor which appears to be related to the decrease in urine production is the mechanical handling of the crayfishes, especially the withdrawal of blood samples at frequent intervals. It can be seen from the data in Tables I and III and the discussion of results that an increase in blood glucose created effects similar to those seen when inulin was injected into the crayfishes. That is, urine production was quite variable and urine sodium increased. The blood glucose data from control animals (Table II) show that there was a spontaneous rise in blood glucose which could only have been due to handling. Further, the urine sodium content rose in all control animals. Therefore, the variability in urine production is closely associated with a rise in blood glucose, which in turn, is possibly due to excessive handling of the crayfishes necessitated by the experimental procedures.

Filtration versus secretion

There is no positive evidence that the crayfish green gland is a secretory kidney. However, there are two principal features of the anatomy of that organ which make it difficult to assign to it the role of a filtration kidney. (1) There apparently is no glomerulus-like structure (the tenuous syncytium of Maluf) against which a hydrostatic pressure head could be applied to effect filtration. Parenthetically, it should be pointed out here that the statement made by Prosser *et al.* (1950, p. 31), "The histological picture (of the crayfish green gland) is not compatible with filtration, however. The coelomic sac and tubules lie in haemocoelic spaces, so that pressure must be similar on all sides, and it is difficult to see how unidirectional pressure could exist as it does in the vertebrate kidney glomerulus," appears to be based on a misconception of the anatomy of the crayfish green gland. As adequately demonstrated by Marchal (1892), Peters (1935) and Maluf (1939) and confirmed by personal observations, all parts of the crayfish green gland are well supplied by branches from the antennal and sternal arteries. These main branches break up into finer and finer branches until eventually the very finest branches become sinusoids. As stated by Peters (1935, p. 359), "Ich konnte beim Flusskrebse die Blutkapillaren bis zu den Zellen verfolgen (Abb. 4, 5 und 6). Fast jede Zelle wird an ihrer Basis vom Blute umspült." Since the vessels are patent, it is possible that even in the smallest sinusoids there is still sufficient arterial pressure to effect filtration. That the sinusoids do respond to pressure may be shown by a quote from Peters (1935, p. 359) concerning the coelomosac, "Wurde unter grossem Drucke injiziert, so traten zahlreiche, sonst fast kollabierte Gefässe in Erscheinung (Abb. 4). Bei normalen Blutdruck scheint ein Teil von ihnen immer kollabiert zu sein." (2) The histological appearance of the green gland suggests abundant secretory activity. Further, the crayfish kidney possibly can secrete Indigo Carmine, as suggested by the fact that that dye becomes con-

centrated in the urine (Maluf, 1941b, and personal observations). Maluf (1941b) utilized these two factors as the main issues when he chose to interpret the results of his experiments as indicating that the crayfish green gland is a secretory kidney. For similar reasons, Burger (1957) was loath to say that filtration occurred in the lobster (*Homarus americanus*) green gland despite good physiological evidence for it. In regard to the first factor, Martin, Harrison and Stewart (1953), working with the terrestrial gastropod, *Achatina fulica*, and Harrison and Martin (1955), working with the cephalopod, *Octopus hongkongensis*, obtained good evidence for filtration in those two molluscs despite the apparent absence from their kidneys of structures analogous to the vertebrate glomerulus. The second factor is hardly worthy of concern, since abundant secretory activity need not exclude the green gland from consideration as a filtration kidney. It is well known that the vertebrate glomerular kidney, although functioning primarily by filtration, excretes many substances by secretion, including Indigo Carmine (Smith, 1951). Further, Cyanol is excreted by the crayfish green gland at low blood concentrations, whereas that dye is not excreted by the vertebrate secretory kidney, except at high blood concentrations (Maluf, 1941b). Finally, a "secretory appearance" does not specify the direction of secretion, *i.e.*, reabsorption could be indicated.

The inulin and glucose excretion data supporting Maluf's (1941b) argument that the crayfish green gland is a secretory kidney need to be examined critically, since they render questionable much of the evidence which otherwise would support a filtration hypothesis. Following is a discussion of Maluf's (1941b) experiments and results reviewed in the light of results and calculations obtained from the present study.

Maluf's conclusion that inulin is secreted rather than filtered derives primarily from the nature of the relationship between urine and blood concentrations. Maluf noted that when the blood inulin levels varied between 100 to 500 milligrams per cent the concentration of inulin in the urine was considerably higher than that in the blood. Between 500 to 1800 milligrams per cent, the U/B ratios were approximately unity. There was an enormous scatter in the experimental points within the lower range, but quite gratuitously, a line was drawn indicating a non-linear decrease which approached unity as a lower limit. The decrease in the inulin U/B ratios with increasing blood inulin concentrations was used to support the thesis that that substance was secreted rather than filtered. Several serious objections can be raised against this interpretation. In the first place, there was too much scatter in the experimental points to justify the drawing of a hyperbola through them. A straight line for the range of blood concentrations between 100 to 500 milligrams per cent would equally well fit the points. Actually, the experimental results presented in this paper (see Fig. 1) show that there is much more basis for this, for when the range of blood concentrations is extended down to less than one milligram per cent inulin, it can be seen that U/B ratios are nearly constant. Certainly there is no indication of the steep hyperbola indicated in Maluf's (1941b) paper.

It should be noted, also, that Maluf's entire argument for the secretion of inulin is based on fallacious reasoning. Assuming that the rate of secretion of inulin is limited because the transport mechanism becomes saturated at high blood

levels (as stated by Maluf, 1941b), it can be shown that the quantity (N) of inulin secreted in unit time is related to the blood level by

$$N = \frac{N'(B)}{K + (B)}.$$

If, as Maluf showed, the volume of urine formed per unit time is constant, then

$$U/B = \frac{U'}{K + (B)},$$

where N' , U' and K are all constants and (B) is the blood concentration. Superficial examination of this equation shows that U/B will be relatively flat over the range of blood concentrations in which (B) is much less than K , and that it will approach zero where (B) is greater than K . An asymptotic approach to a lower limit of one will be realized only if filtration is assumed *in addition* to secretion. In fact, a kidney that excretes inulin both by filtration and secretion could produce the pattern found experimentally; that is, inulin U/B ratios greater than one when the blood inulin concentrations are low, followed by a decrease to unity at high blood concentrations. While this possibility must be kept in mind, Maluf's experiments at high blood inulin levels are open to criticism of such a nature as to render the significance of his results suspect. The molecular weight of inulin is about 5000 (Westfall and Landis, 1936). Assuming that it is non-penetrating (which, of course, is implicit in its frequent use for measurement of extracellular fluid volume), the use of large quantities will raise the colloid osmotic pressure (COP) of the blood. Thus, Picken (1936) reported that the COP of crayfish blood averages about 15 cm. of H_2O . An inulin concentration in the crayfish's blood of 500 milligrams per cent would increase the COP of the blood by 23 cm. of H_2O . Even assuming that the animal could make compensatory adjustments in blood pressure and extracellular fluid volume, these adjustments could be effective only over a limited range. The fact that U/B inulin ratios begin to decrease at about 500 milligrams per cent, and remain near unity at blood levels of 1000 to 1800 milligrams per cent may represent little more than renal failure of some kind due to abnormal elevation of blood COP. If a lower molecular weight is used (Hodgman, 1955), the increase in COP would be even greater. Thus, before Maluf's (1941b) data could be used to support any hypothesis, a rigorous investigation should be undertaken to show that the antennal gland functions normally under conditions of high blood inulin levels. The best that can be said in favor of a secretory process is that the organ might be excreting inulin by a combination of filtration and secretion. However, the introduction of an uncontrolled variable (colloid osmotic pressure) at high blood inulin levels makes the evidence for secretion tenuous. Since either secretion or filtration would yield U/B inulin ratios practically independent of the blood concentration of that substance when the latter is low, the inulin data in this range provide no support for either mechanism of urine formation.

There are, however, other data which are better explained on the basis of urine formation by filtration followed by reabsorption. The data in Table II show that glucose usually is not excreted in the urine, but if the blood concentration is

sufficiently high (perhaps about 200 mg. %), it will begin to appear. Thus, glucose may behave in the crayfish as it does in vertebrates with glomerular kidneys, *i.e.*, as a threshold substance. For some reason, Maluf (1941b) used xylose rather than glucose, and his data seem to indicate either no threshold, or a very low one. However, when his figure showing blood xylose levels is examined, it can be seen that the initial blood concentrations were invariably much higher than those tabulated with the urine concentrations; in fact, they were always higher than 200 milligrams per cent, although, as in the glucose experiments presented in this paper, they fell very rapidly. Thus, the blood concentrations shown in Maluf's table on xylose excretion are misleading in that they represent, in every case, values much lower than the level immediately following injection. It is not possible to compute clearances or reliable thresholds in the face of blood levels that fall (and rise) as rapidly as seen in Maluf's and the present investigation. The glucose (xylose) appearing in the urine may all have been excreted when the blood concentration was very high and the computation of a clearance on the basis of "average" blood concentrations may actually obscure this information. This is certainly the case in the present experiments with glucose. Serial sampling showed (Table II) that urine glucose is zero prior to the injection of exogenous glucose, and where glucosuria occurs, it always does so in the period following the peak in blood glucose and returns to zero when the level of that substance in the blood falls.

Moreover, the experiments with phlorizin are difficult to rationalize on the basis of secretory activity. The consistent and sustained glucosuria following the administration of that drug must indicate that glucose normally appears in a presumptive urine from which it is subsequently reabsorbed. The highest glucose U/B ratios following phlorization were 3 to 4, which brings them into the range of the inulin U/B ratios, and hence supports the conclusion that the latter can be used to measure fluid turnover in the crayfish green gland.

Therefore, despite the apparent absence from the crayfish green gland of a well-defined site for filtration, the facts that inulin can be excreted and that phlorizin causes glucosuria lead the authors to conclude that filtration (in the limited sense explained at the beginning of this discussion) does occur in that organ. In addition, it is quite probable that filtration is followed by the reabsorption of water and a large quantity of solute.

SUMMARY AND CONCLUSIONS

1. Inulin is excreted by the green gland of the crayfish, and within the range of blood inulin levels used in the experiments presented here, the inulin urine/blood ratios were always greater than one.

2. Glucose normally is not excreted by the crayfish green gland. However, if the blood glucose level exceeds about 200 milligrams per cent, it usually appears in the urine. Further, after thirty or more hours of handling (see paragraph 3 below), many crayfishes began to excrete glucose.

3. Handling (*i.e.*, injection of material, and, especially, serial withdrawal of blood samples) often resulted in erratic urine flow, concentration of the urine (reflected by large increases in inulin and sodium concentrations in small, less than 10-microliter, urine samples).

4. Phlorizin caused an immediate and prolonged glucosuria at all dosage levels tested (12 to 102 mg./Kg.).

5. Despite the absence of a well-defined site in the crayfish green gland at which filtration (in a limited sense, defined in the text) can occur, the authors conclude that because inulin is excreted and glucose causes glucosuria, filtration does occur in that organ. Furthermore, the filtration is accompanied by the reabsorption of salts and possibly water.

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THE EFFECTS OF GLUCOSAMINE HYDROCHLORIDE ON THE DEVELOPMENT OF *DROSOPHILA MELANOGASTER*¹

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An unusual type of abnormality in *Drosophila* was encountered during a series of feeding experiments involving glucosamine hydrochloride. These abnormal pupae resembled the *cryptocephal* recessive lethal described by Hadorn and Gloor (1943). Since a high incidence of abnormal individuals was recovered after treatment, and none of the other sugars tested produced morphological abnormalities, a more detailed examination of the conditions responsible for this effect, as well as a histological examination of the abnormalities, was undertaken. This report summarizes the observations of the effects produced by feeding glucosamine hydrochloride to larvae of *Drosophila melanogaster*.

MATERIALS AND METHODS

The *tu^w* mutant strain which develops melanotic tumors in the caudal fatbody (Wilson *et al.*, 1955; Rizki, 1957) and the *Ore-R* wild type strain have been used in the present study. The abnormal pupae were first noted in experiments in which larvae were removed from Cream of Wheat-Fleischmann's yeast medium and placed on paper strips moistened with a solution of glucosamine hydrochloride. This same method was therefore used in subsequent experiments, as well as merely adding the test substance to the medium on which larvae were feeding. Each group of larvae was collected during a two- to three-hour interval, and larval ages are counted from the time of occlusion from the egg. All experimental material was maintained at 23°–25° C. in an incubator.

In the series of starvation experiments, the timed larvae were removed from the food dishes and washed in a series of changes of a saturated solution of NaCl, a 2% solution of NaOCl, followed by at least six rinses of distilled water. The larvae were then placed on tissue paper strips (Kleenex brand) in sterile petri dishes with filter paper under the covers to prevent the escape of the larvae from the dishes. Distilled water was used to moisten the paper strips in the control dishes, and the test solutions were pipetted onto the paper strips in the experimental dishes. Conditions were standardized throughout the study by using four layers of paper, size 2.5 × 2.5 inches, with 2 ml. of test solution for each group of 15–20 larvae. In some experiments sterile cellulose pulp was used as the crawling site for the larvae in place of paper strips. In other experiments the sugar solutions were added to Cream of Wheat-Fleischmann's yeast medium. In these cases the larvae were washed as above and approximately the same area of the petri dish was covered with the food as the size of the paper strips used in the starvation ex-

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periments in order to maintain conditions as similar as possible throughout the study.

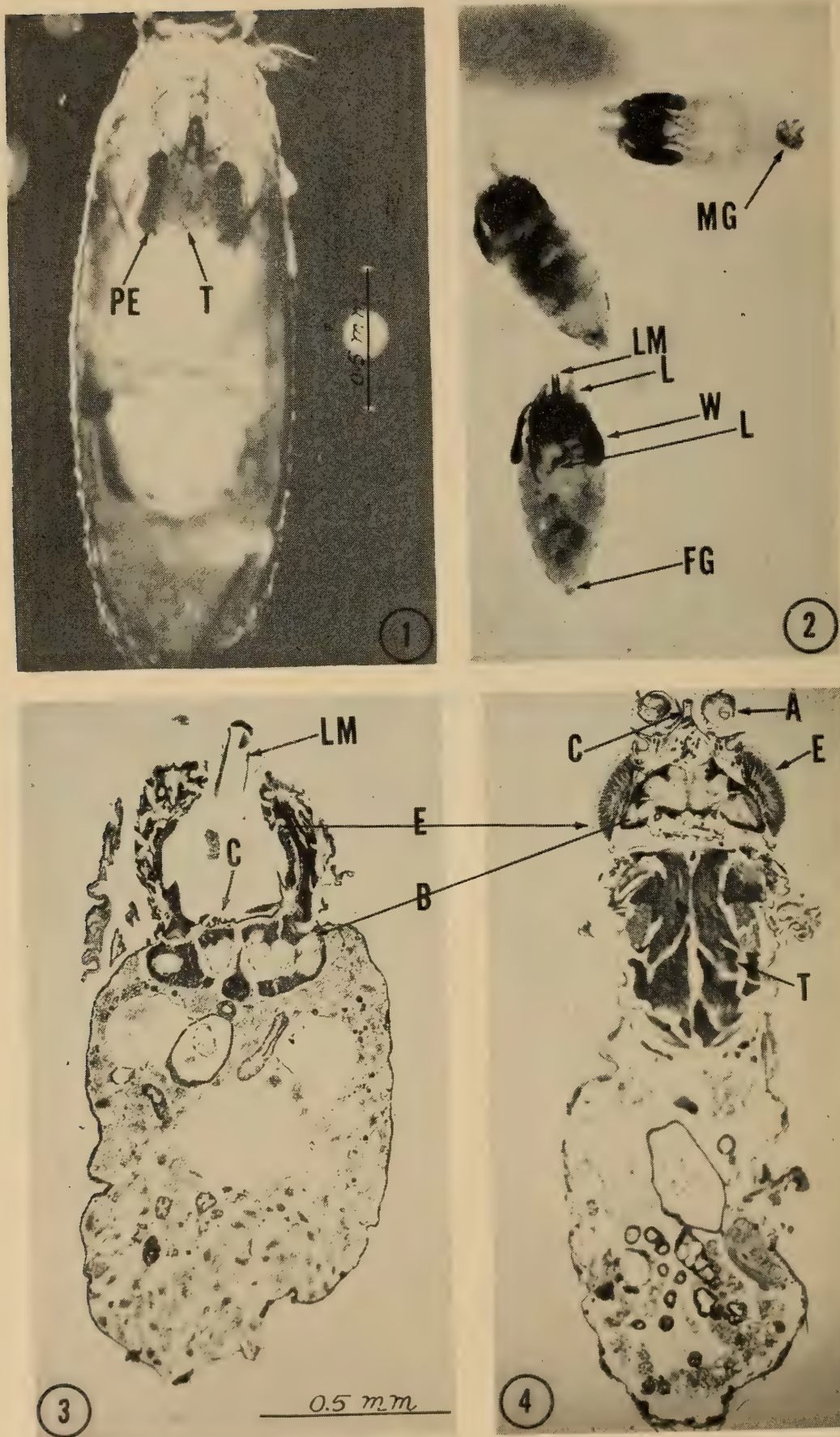
The abnormal pupae were dissected in Waddington solution. The histological material was prepared by fixing larvae and pupae in Carnoy solution, embedding paraffin, and staining the sectioned specimens with Azure B (Flax and Himes, 1952).

RESULTS

The typical pattern of the abnormality induced by feeding glucosamine hydrochloride involved the disproportionate sizes and relative positions of the structures of the head and thorax. The most striking feature of the abnormal pupae was the reduced size of the eyes which nevertheless developed normal pigmentation (Fig. 1). Examination of pupae after removal of the puparium showed that the cephalic structures were contained in the thorax. The thoracic structures, including the legs and the wings, have developed, their distortion in position being a result of the presence of the head within the thorax. In some cases the legs appeared shorter than normal and were not fully stretched (Fig. 2). The larval mouthparts were still attached to the anterior end of the thorax, and many of the puparia were unusually elongated. The degree of contraction of the larval cuticle had been less in these cases. In some pupae the bristles were well formed but in others the development of these structures, particularly in the posterior region of the body, had been delayed. None of the abnormal pupae hatched, and sectioned material from these pupae was subsequently examined.

Stained preparation of the sectioned pupae revealed that eversion of the imaginal discs of the imaginal complex had occurred but these developing head structures did not emerge from the thorax. Figures 3 and 4 are frontal sections passing through the brain in an abnormal pupa and a normal specimen, respectively. In the former, the brain is located in the anterior region of the abdomen. Comparison of these sections indicates the degree of morphogenetic displacement which occurs in the brain mass. In the normal pupa, as the cerebral ganglion is pushed forward, the anteriolateral parts of the brain are displaced caudolaterad and the first optic stalks assume a lateral position to the brain. In the abnormal specimens the antennae and palpi appear normal with respect to their morphological development but they are found lodged in the area enclosed by the eyes. The carina is not properly formed. The gonads of the abnormal pupae are well developed and no apparent abnormality of the external genitalia would be detected. Eversion of the wings and legs has been complete. A parasagittal section of an abnormal pupa is presented in Figure 5 to illustrate the development of the thoracic muscles, tergites, and bristles. In this photograph, the compound eyes, although failing to emerge from the thorax, show well formed rhabdomeres and crystalline cones, and the setae are normal in appearance. Several pupae were found which had dwarf heads, and improper wing eversion was apparent in a few adults.

The first group of abnormal pupae was recovered during a survey of nutritional factors which were being tested for their effects in the mutant strain, *tu^w*. For these experiments larvae 65 hours of age were removed from food and placed on paper moistened with a 10% solution of each test substance. Larvae remained in these dishes until after pupation, and scoring was completed as the adults hatched. Under these conditions, glucosamine hydrochloride was the only chemical



FIGURES 1-4.



FIGURE 5. Parasagittal section of a glucosamine-induced phenocopy of *crc* in the *Ore-R* strain showing the detailed structures of the eye, E; optic lobe, O; longitudinal thoracic muscles, T; muscles of the appendages, L. A large portion of the brain is in the abdomen demarcated by A.

which induced any noticeable morphological changes. The list of substances tested included N-acetyl glucosamine, thiamine hydrochloride, fructose, glucose, lactose, starch, mannitol, salicin, casein hydrolysate, nucleic acid hydrolysate, glycogen, melibiose, trehalose, ribose, sorbose, inulin, sucrose, maltose and galactose. *Drosophila* larvae 65 hours of age were removed from food and placed on paper moistened with distilled water. These larvae completed their development and normal adults were obtained. Repetition of these conditions with glucosamine hydrochloride yielded the same type of abnormalities each time, and this method

FIGURE 1. An atypical pupa within the puparium obtained after feeding glucosamine hydrochloride to third instar larvae of the *Ore-R* strain. The concave discoid pigmented eye, PE, is located in the thorax; the arrow, T, is pointing to the scutellar bristles of the thorax.

FIGURE 2. Atypical imagoes removed from the puparium (*tu^w*). Note the absence of the cephalic region while the thorax and abdomen with their associated structures are well formed. Female genitalia, FG; leg, L; male genitalia, MG; wing, W; the larval mouthparts, LM, still attached to the anterior region of the thorax. The legs are not fully stretched.

FIGURES 3 AND 4. Comparison of frontal sections of a glucosamine-induced abnormal pupa (*tu^w*) and a normal *tu^w* imago. In the abnormal specimen in Figure 3 the brain is located in the abdomen and the eye discs are flanking the inner lateral sides of the thorax forming a cavity. Larval mouthparts, LM; carina, C; antenna, A; eye, E; muscles of flight in the thorax, T.

was used to test 1%, 5%, 10%, and 20% solutions of glucosamine hydrochloride and N-acetyl glucosamine in succeeding experiments. In addition these same solutions were added to a thin layer of food covering approximately the same area of the dish as the size of the paper strip.

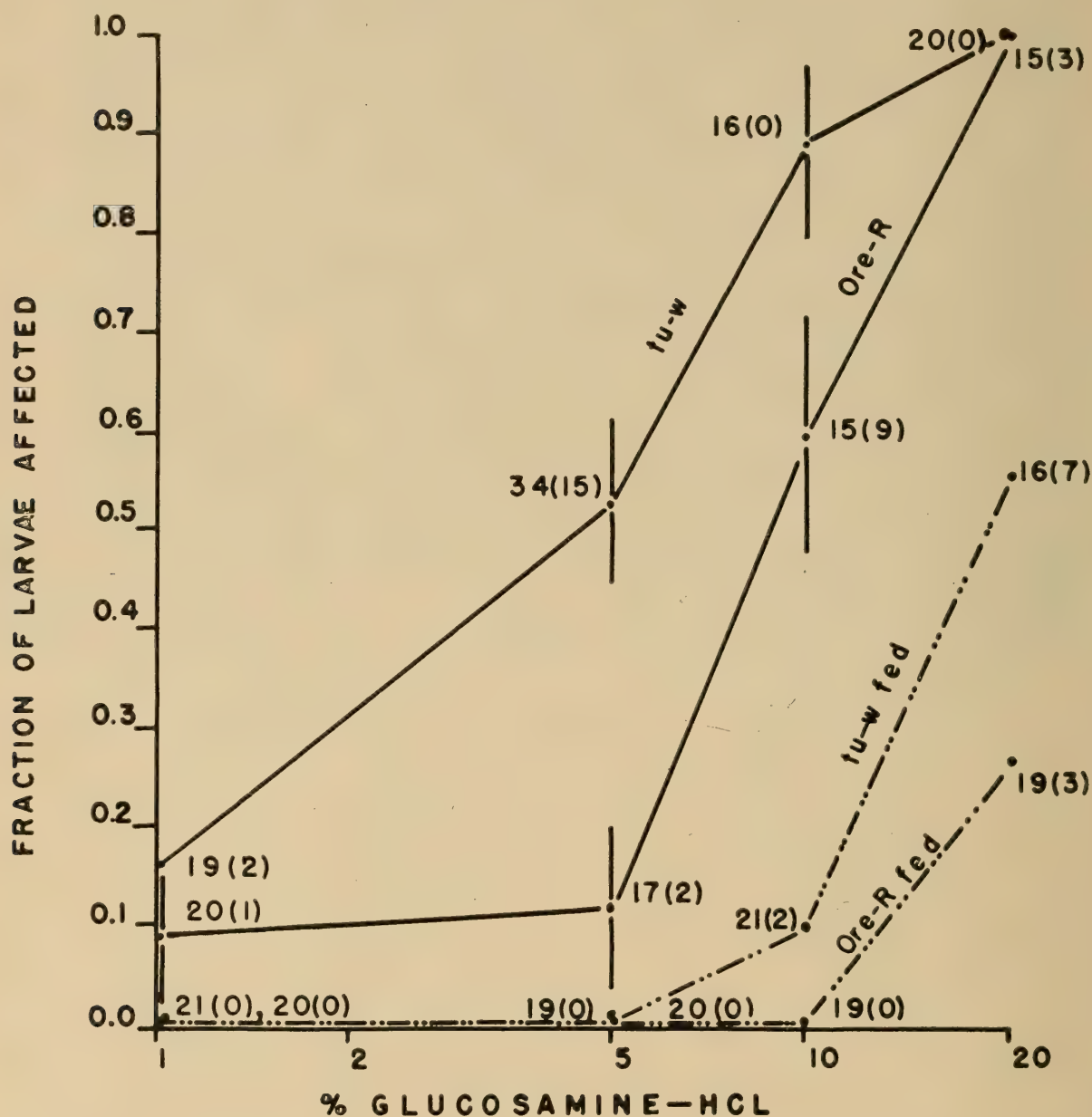


FIGURE 6. The yield of abnormal pupae and larvae in the *tu^w* and *Ore-R* strains obtained after feeding varying concentrations of glucosamine hydrochloride. The solid lines represent starvation conditions in the *tu^w* and *Ore-R* strains, and the vertical lines on these two curves indicate standard deviations. The results on the fed larvae are represented by dotted lines. All of the larvae used in this experiment (#756) came from one collection period. The fraction of larvae affected includes the total effect of feeding glucosamine hydrochloride, that is, abnormal pupae as well as larvae which died before pupation. At each point on the graph, the number preceding the parenthesis is the sample size which is followed by a number in parentheses giving the count of the pupae at this point with the *cryptocephal* phenocopy. For example, the higher per cent of phenocopies in the *tu^w* strain under starvation conditions was obtained with 5% glucosamine hydrochloride; no phenocopies were obtained in the *tu^w* strain with 10% and 20% glucosamine hydrochloride as the larval lethality approached 100%.

None of the *tu^w* larvae survived in the 20% solution of glucosamine under starvation conditions, and very few of the *Ore-R* larvae pupated. Many of the larvae crawled off the paper at this concentration, so the high mortality is presumably the combined effect of the glucosamine as well as desiccation. Behavior of this type was not noticed in the dishes with the lower concentrations of glucosamine. Combining the number of dead larvae and abnormal pupae obtained, the fraction of response to glucosamine hydrochloride can be graphically represented, thus indicating the difference between the two strains of flies. Figure 6 illustrates the results of one experiment on larvae taken from the same collection period. Direct comparison between larvae on food and those on paper strips cannot be made, but the difference most probably represents the dilution factor of the food since the same amount of solution was added to the food surface as that added to the paper in the dishes. The type of abnormalities induced was the same under conditions of feeding and starvation, as well as in the two strains of flies. The optimum concentration for the production of abnormal pupae was 5% for the *tu^w* strain and 10% for the *Ore-R* strain when larvae were removed from food at 65 hours of age, and these were the concentrations which were used in the experiments from which material was fixed and sectioned. In the present study, a total of 276 abnormal pupae was obtained from the *tu^w* strain and 99 from the *Ore-R* strain. Under optimum conditions of treatment the per cent of abnormal pupae in both strains was as high as 60%.

Samples of glucosamine hydrochloride obtained from three sources were used (Nutritional Biochemicals Corp.; California Corporation for Biochemical Research; Krishell Laboratories) and all produced the same type of abnormalities. The dosage response decreased with the age of the solution of glucosamine hydrochloride, so only freshly prepared solutions were used.

DISCUSSION

The typical pattern of abnormality obtained after feeding glucosamine hydrochloride to *Drosophila* larvae is the same as that reported by Hadorn and Gloor (1943) for the recessive lethal which they termed *cryptocephal*, *crc*. This name appropriately described the characteristic feature of the glucosamine-induced abnormal pupae with the head structures contained in the thorax. Histological examination of the abnormal specimens has shown that the cephalic complex has undergone development and eversion, and the principal morphological difference appears to be the displacement of structures resulting from the lack of emergence of the head. A similar developmental upset was obtained in two stocks, the wild type *Ore-R* and the *tu^w* mutant strain, although a higher percentage of abnormalities was induced in the latter strain under the same conditions of treatment. Comparing pupation time in the two stocks, there is an average delay of at least twelve hours in the *tu^w* strain. Therefore the difference in response to glucosamine may reflect a difference in developmental stage since the larvae from both stocks were of the same age at the time of treatment. A survey of the effects produced by glucosamine hydrochloride when offered at different ages throughout larval life in both stocks will have to be undertaken before this question of difference of response can be answered.

Gloor (1944) reported lack of emergence of the head in some of the pupae of a wild stock of *D. melanogaster* by using temperature shocks during the prepupal

period shortly before the normal time of emergence of the head. The use of glucosamine hydrochloride as a phenocopic agent may provide a useful tool in studying the physiological mechanism of emergence of the head in the developing pupa. The biological role of the aminosugars has been recently reviewed by Kent and Whitehouse in their monograph (1955). Glucosamine and N-acetylglucosamine are components of the polysaccharide polymer, chitin. Complexes of chitin and protein are important constituents of insect cuticle. The incomplete contraction of the cuticle, apparent in some *Drosophila* specimens after feeding glucosamine hydrochloride, may suggest this system as a point of interference. The reported inhibition of anaerobic glycolysis and O₂ uptake by glucosamine in leucocytes (Alonso, 1959) also suggests further examination for such effects in *Drosophila* larvae and pupae. The ease with which abnormal pupae were obtained under starvation conditions was utilized to obtain as large a number of specimens as possible for the present survey. However, this restriction of starvation may not be desirable for an analysis of the mechanisms underlying the formation of the abnormalities since the histological condition of the abnormal specimens obtained in starvation experiments was inferior to that in the fed experiments.

SUMMARY

Abnormal pupae were obtained after feeding glucosamine hydrochloride to larvae from two stocks of *Drosophila melanogaster*, the *Ore-R* wild type strain and the mutant melanotic strain, *tu^w*. The characteristic feature of the abnormality was the position of the head within the thorax, thus suggesting the use of glucosamine hydrochloride as a phenocopic agent to produce individuals resembling the recessive lethal *cryptocephal*.

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INTRACELLULAR DIGESTION AND HYDROLYTIC ENZYMES IN THE PHAGOCYTES OF PLANARIANS¹

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It has been established that, in those species studied, intraluminal digestion does not occur in triclad flatworms (Willier, Hyman and Rifenburgh, 1925; Jennings, 1957), but that digestion takes place within food vacuoles in the phagocytic cells of the gastrodermis. A number of investigators have observed the disintegration of ingested food particles within these cells (Arnold, 1909; Saint-Hilaire, 1910; Jacek, 1917; von Levitzow, 1943), but the methods they employed did not permit elucidation of the mechanisms involved except in a most general way. Kelley (1931), studying the intracellular digestion of nucleoprotein in *D. dorocephala* by means of the Feulgen reaction with and without acid hydrolysis, showed that the ingested material was hydrolyzed within the first two hours.

To our knowledge, no previous study has characterized the hydrolytic enzymes in the phagocytes of planarians. The present report considers the relationship of three such enzymes to current concepts of intracellular digestion.

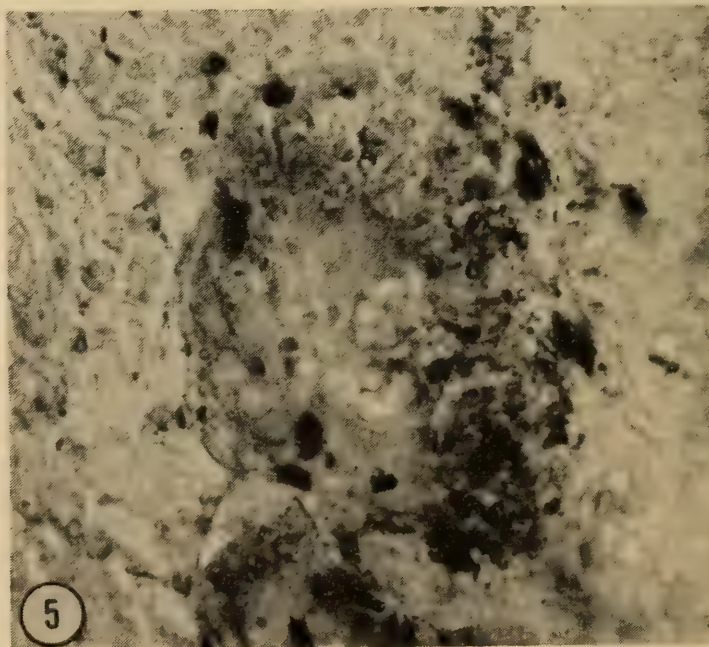
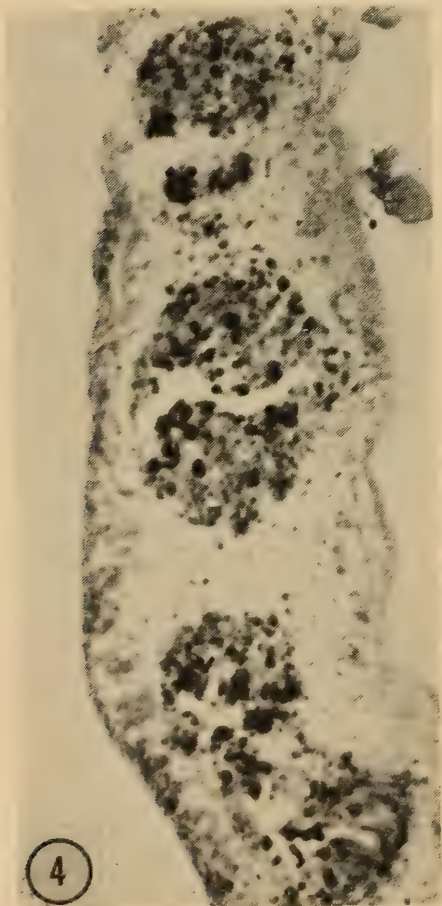
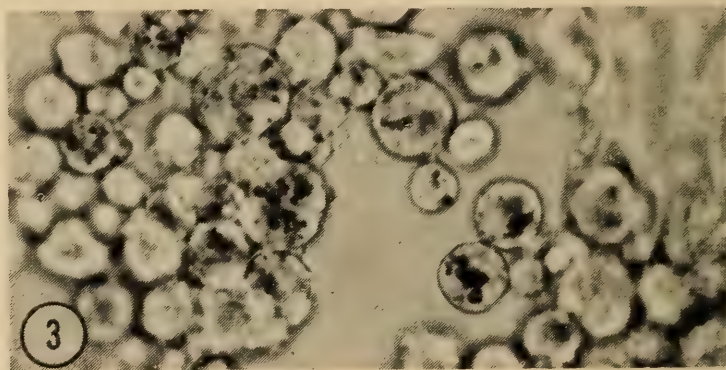
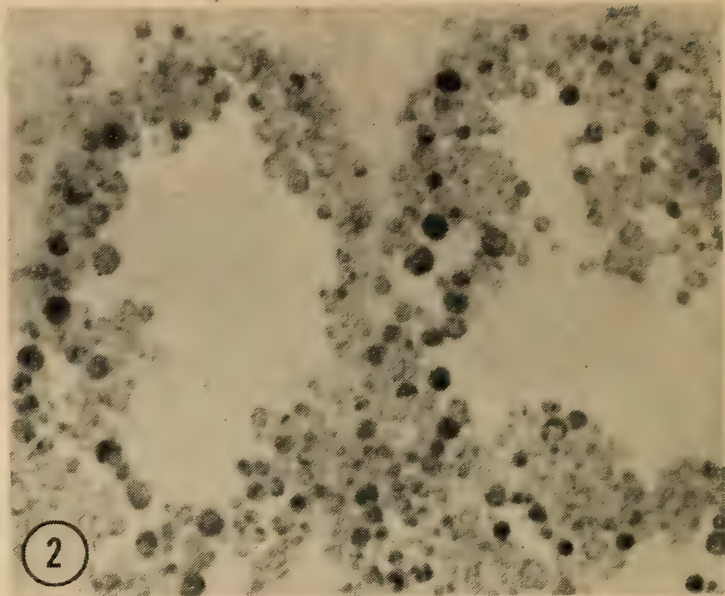
MATERIALS AND METHODS

Specimens of *Dugesia dorocephala* and *D. tigrina* were used for this study. The animals were maintained as stock cultures at 18° C. and fed routinely once every ten days on minced rat or mouse liver. Experimental animals were isolated in fingerbowls and starved for at least three weeks. Some of these were fed raw rat or mouse liver, and others boiled liver to determine if enzyme activities in raw food were transferred to the gut. No differences in enzyme activities between animals fed raw or boiled liver were demonstrable by the staining methods employed. The worms were observed during feeding; post-feeding times were determined from the moment the animals ceased feeding.

For the demonstration of those enzyme activities surviving chemical fixation, specimens were initially fixed in warm (37° C.) Baker's calcium-formalin for 5 minutes to prevent discharge of the gut contents. They were then placed in calcium-formalin at 4° C. for 24 hours, washed for 5 minutes in distilled water and cut on a CO₂-freezing microtome at 8–10 μ . Material thus prepared was incubated for acid phosphatase activity by a slight modification of the method of Gomori (1952). Sections were incubated for 15–30 minutes in a 0.1 *M* 2-glycerophosphate and lead nitrate mixture buffered to pH 5.0 with 0.05 *M* acetate buffer.

Aminopeptidase activity was visualized by the method of Burstone and Folk (1956). Animals were placed on Monel grids and frozen in isopentane chilled by liquid nitrogen to –160° C. After quenching, the worms were dehydrated for 24

¹This investigation was supported by a research grant, No. RG-5483, from the United States Public Health Service.



FIGURES 1-5.

hours in a vacuum (10^{-3} mm. Hg) at -28° C. and infiltrated for 30 minutes with $54-56^{\circ}$ C. paraffin. Paraffin sections cut at 8μ were incubated at 37° C. in substrates consisting of either L-leucyl- β -naphthylamide (LNA) or L-alanyl- β -naphthylamide (ANA) buffered to pH 7.1 with 0.2 M Tris buffer. Control sections were incubated in a solution of β -naphthylamine in 0.2 M Tris buffer. Garnet GBC or Fast Corinth Salt V were employed as simultaneous couplers in the incubation medium.

β -glucuronidase activity was demonstrated by the method of Billett and McGee-Russell (1955). It was not possible to achieve a precise intracellular localization of the final ferric-8-hydroxyquinoline crystals with formalin-fixed frozen sections. As an alternative, the entire worm was placed in quinolyl-8-glucuronide at pH 4.2-4.5 and incubated in the presence of a saturated ferric-8-hydroxyquinoline solution at 37° C. for 24 hours, following which they were washed, first in distilled water, then in oxalate buffer and finally mounted *in toto* in glycerine jelly.

RESULTS

Histology of the gastrodermis

The gastrodermis consists of a single layer of epithelial cells resting on a delicate basement membrane. Two distinct cell types are present. The phagocytic cell is the larger, the more numerous and contains a basal nucleus and a free end that protrudes into the gut lumen. The cytoplasm is basophilic and has a varying number of eosinophilic inclusions that progressively disappear after feeding. These presumably represent the contents of food vacuoles undergoing digestion (Willier, Hyman and Rifenburgh, 1925).

The second cell type is smaller and less columnar than the phagocyte although its shape varies considerably, depending on the nutritive state of the animal. It has been variously described as a storage, gland or spherical cell and contains intensely staining eosinophilic bodies generally considered to be a protein reserve. The morphology of these two cell types throughout the digestive process has been considered in detail by Willier, Hyman and Rifenburgh (1925).

Acid phosphatase

No activity was detected in any of the cells comprising the gastrodermis of starved animals (Fig. 1). Due to the presence of diffuse brown-black pigment

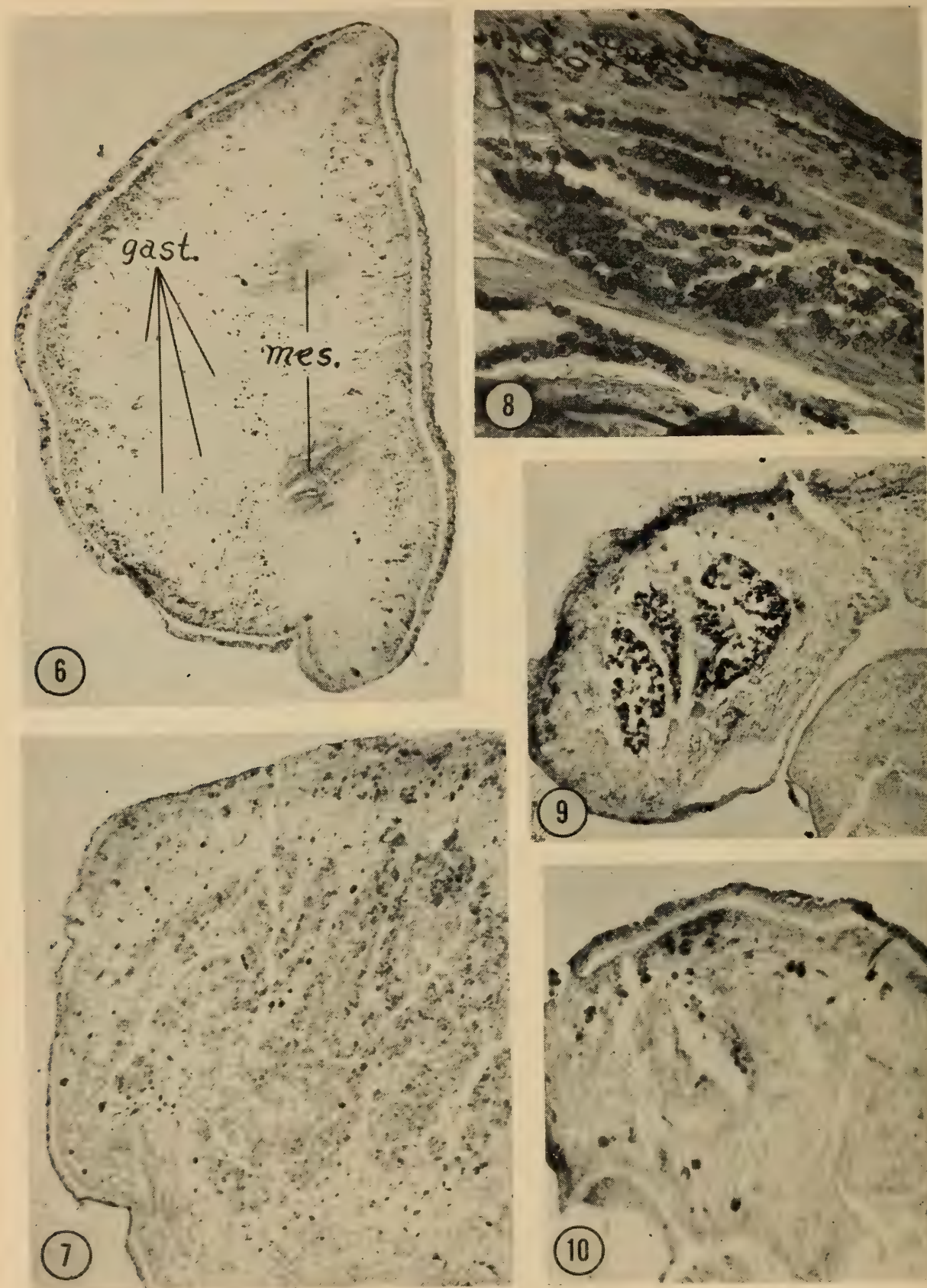
FIGURE 1. *D. dorotocephala*. Starved (14 days) animal showing three segments of digestive tract (arrows). There is no activity in the gastrodermis. A number of pigmented cells are seen, as well as abundant pigment in the dorsal epidermis. Gomori lead sulfide reaction incubated for 30 minutes at pH 4.5. $100\times$.

FIGURE 2. *D. dorotocephala*, gastrodermis 6 hours post-feeding. There is acid phosphatase activity in many of the food vacuoles but not all cells are equally reactive. $420\times$.

FIGURE 3. Acid phosphatase activity in fragments of gastrodermis from *D. tigrina* fed 5 minutes previously. There is a distinct precipitation of lead sulfide within the vacuoles of the phagocytic cells which in some cases have become rounded during maceration. $620\times$.

FIGURE 4. Acid phosphatase activity in the gastrodermis of *D. dorotocephala* 24 hours after feeding. Notice the intense staining in the food vacuoles of the phagocytic cells. A weaker reaction is evident throughout the gastrodermis. $100\times$.

FIGURE 5. Section of gastrodermis from *D. dorotocephala* 10 days after feeding. There is some acid phosphatase activity in the cytoplasm but no food vacuoles are present. Several pigment cells are evident. $420\times$.



FIGURES 6-9.

throughout the animal, controls were necessary to distinguish such pigment from the similarly colored deposits of lead sulfide in a positive enzyme reaction. Sections serving as controls were either heated in a water bath at 60° C. for 45 minutes or incubated in buffer without glycerophosphate.

Some enzymatic activity could be detected in sections of animals fed 30 minutes previously. The gastrodermis of animals killed six to twelve hours after feeding showed distinct acid phosphatase activity. Activity was localized within vacuoles formed in the phagocytic cells (Fig. 2), but the intensity of the reaction varied considerably from vacuole to vacuole. Such differences in enzyme activity are presumably dependent on the amount of material phagocytized, the length of time the food has been in the vacuole and, hence, the extent of digestion taking place in the vacuole at the time of fixation.

Attempts were made to visualize the intracellular distribution of acid phosphatase during formation of the food vacuoles. Since frozen sections did not provide optimal preservation of cytological details, a series of small animals, fed approximately 30 minutes previously, were squashed on chilled coverslips and immediately fixed in cold calcium-formalin. These preparations were then incubated in buffered (pH 4.8) glycerophosphate and acid phosphatase activity visualized by precipitated lead sulphide. As early as five minutes after initiation of feeding, acid phosphatase could be demonstrated within newly forming food vacuoles (Fig. 3). During formation of the food vacuole, the sites of enzyme activity are discrete but of varying size depending on the physiological activity of the vacuole. Four to six hours after feeding, most vacuoles showed such intense acid phosphatase activity that the entire vacuolar space was filled by a lead sulphide precipitate. No cytoplasmic enzyme activity could be detected at this time.

Twenty-four hours after feeding, intense acid phosphatase activity occurred throughout the gastrodermis (Fig. 4). While staining was still most striking within the food vacuoles, a reaction also occurred in the cytoplasm of the columnar cells. In all instances, this was limited to the gastrodermis with no staining demonstrable in the mesenchyme (Fig. 4). This activity, both in vacuoles and cytoplasm, persisted up to 48 hours, following which a gradual diminution became evident.

One week after feeding, a few cells in gastrodermis showed slight staining for enzymatic activity (Fig. 5).

FIGURE 6. *D. dorocephala*, starved for two weeks and incubated in L-leucyl- β -naphthylamide for aminopeptidase activity. There is no staining in the gastrodermis (gast.) but two regions in the mesenchyme (mes.) show enzyme activity. Numerous pigment cells are present throughout the section, especially in the dorsal epithelium. Photographed in combination with Corning narrow band filters, Nos. 3486, 9782 and 5120. 100 $\times$.

FIGURE 7. Aminopeptidase activity in the gastrodermis of *D. dorocephala* 30 minutes after feeding. Staining is not uniform but its localization within vacuoles is evident. 210 $\times$.

FIGURE 8. Aminopeptidase activity localized within vacuoles in the gastrodermis of *D. dorocephala* 12 hours after feeding. There is no reaction in the cytoplasm of the phagocytic cells. 460 $\times$.

FIGURE 9. Aminopeptidase activity in the gastrodermis of *D. dorocephala*. The picture is typical of the reaction in animals 24 to 48 hours after feeding. 160 $\times$.

FIGURE 10. *D. dorocephala* starved for 10 days. No aminopeptidase activity is present. 160 $\times$.

Aminopeptidases

Although both leucyl- and alanyl-derived substrates were employed, it was impossible to distinguish differences in activity between the two substrates. The pH optima (7.0–7.6) and optimal incubation times for both substrates were also identical. Only slight activity was obtained with both substrates at a more acid pH (4.5–5.0).

There was some aminopeptidase activity in the mesenchyme of starved animals although none could be detected in the gastrodermis (Fig. 6). The presence of enzyme in the mesenchyme of starved animals was probably related to a lowered nutritional state since an increase in enzyme activity in the mesenchyme of animals starved for two, four or six weeks, respectively, was always observed. This suggests that a mechanism is present outside the gastrodermis for utilization of the animals' tissues as a food reserve. The ability of planarians to withstand long periods of starvation with a corresponding reduction in body size is well known.

Aminopeptidase activity was detected in the gastrodermis 30 minutes after feeding (Fig. 7). The staining appeared strongest in those cells possessing the largest food vacuoles and, hence, undergoing most active phagocytosis.

Twelve hours after feeding, nearly all the phagocytic cells of the gastrodermis contained food vacuoles which stained positively for aminopeptidases (Fig. 8). Staining was most intense 24 to 48 hours after feeding (Fig. 9), following which there was a rapid decline in activity throughout the gastrodermis.

Seven days after feeding (Fig. 10), no reaction for aminopeptidase was obtained in the gastrodermis. At this stage, some cells in the mesenchyme did possess a positive reaction for these enzymes (Fig. 10). This appears to support the observation of Jennings (1957), that phagocytosed material can pass back into the mesenchyme for subsequent digestion.

Beta-glucuronidase

The method originally suggested by Billett and McGee-Russell (1955) for the demonstration of β -glucuronidase was not satisfactory for fresh-frozen sections of planarians. Even tissue from animals fixed for three hours in cold neutral formalin did not satisfactorily withstand incubation in quinolyl-8-glucuronide at pH 4.0–4.5. Since intracellular localization of the enzyme could not be obtained in sections, it was decided to use the *in toto* incubation method employed by Billett and Mulherkar (1958) for the chick embryo. Small, recently fed worms were fixed in cold neutral formalin for three hours. They were then thoroughly washed in several changes of cold distilled water and incubated in quinolyl-8-glucuronide for 24 hours at 37° C. as previously described. Control animals were incubated in substrate to which 0.0005 *M* potassium acid saccharate had been added as an inhibitor. The presence of ferric-8-hydroxyquinoline crystals could be detected in the gut of animals 24 or 48 hours after feeding. No similar reaction was obtained in the gut of starved animals. Although the technique employed for β -glucuronidase was not satisfactory from the standpoint of intracellular localization, it did demonstrate the presence of β -glucuronidase activity parallel in time to the appearance of acid phosphatase and aminopeptidase.

DISCUSSION

While a considerable amount of investigative work has been undertaken on intracellular digestion following phagocytosis or pinocytosis in organisms such as *Ameba*, little is known about the digestive enzymes present in other invertebrates (Kitching, 1956). In the present reports, the localization of specific hydrolytic enzymes in some fresh-water triclads has been demonstrated in relation to the food vacuoles formed within phagocytic cells in the gastrodermis.

The acid environment within food vacuoles has long been known and it has been assumed that this is related to the function of the digestive process. The pH optima of acid phosphatase and β -glucuronidase are approximately the same (4.2–4.6). Jennings (1957) has obtained a value of pH 4.6 in the food vacuole of the triclad, *Polycelis*, six hours after feeding. Our own data (unpublished) show similar values for *Dugesia*.

It has not been sufficiently emphasized that aminopeptidase activity, identified by the methods employed here, does not *ipso facto* demonstrate the leucine aminopeptidase considered as a single enzyme by Smith and Spackman (1955). Other peptidases are capable of splitting leucyl peptides (Fleisher, 1954) and it is therefore necessary to assume caution regarding the degree of specificity obtained by the present methods. The similarities, both in the degree and distribution of enzyme activity as well as pH optima, obtained with the substrates LNA and ANA in the present investigation, suggest that we are dealing with such a lack in specificity. Recently, Waldschmidt-Leitz and Keller (1957) have demonstrated that "leucine" aminopeptidase is a mixture of peptidases and does not represent a specific enzyme. The histochemical significance of this problem has been considered in detail by Glenner *et al.* (1959).

It is interesting to consider the possible mechanisms responsible for release of enzymatic activity following feeding. The observation that the three enzymes studied here appear *after* the majority of food vacuoles have formed suggests a secretion of enzymes from the cytoplasm into the vacuole. An alternative mechanism would call for the *de novo* synthesis of enzymes within the forming vacuole but then one would expect to find enzymatic activity appearing along with the earliest formation of the food vacuole. Some evidence for the secretion of enzymes from cytoplasm into vacuoles has come from observations of the microkinetospheres of certain cell variants of HeLa cultures (Rose, 1957). The localization of acid phosphatase activity throughout the gastrodermis 24 to 48 hours after feeding indicates that the cytoplasm of these cells possesses the capacity to synthesize the enzyme. There is evidence from the present observations that aminopeptidase is similarly localized in the cytoplasm of cells. In starved animals, for example, activity was found in the mesenchyme and the possibility that such sites may represent localized digestion of the animals' own tissue—perhaps for nutritive purposes—has been mentioned.

The three enzymes studied represent a class of six or more soluble acid hydrolases collectively associated with a group of cytoplasmic particles termed "lysosomes" by de Duve (1959). Although the lysosome concept has been investigated most thoroughly in the liver cell, there is increasing evidence that these particles may be concerned with localized phenomena related to the acid digestion

of cells in general. Evidence for this is available from observations on *Ameba* (Bennett, 1956); spleen macrophages (Palade, 1956) and, most conclusively, in "athrocytosis" phenomena in kidney cells (Straus, 1957).

SUMMARY

1. The presence of the three hydrolytic enzymes, acid phosphatase, aminopeptidase and β -glucuronidase have been demonstrated in relation to digestion phenomena in *Dugesia dorotocephala* and *D. tigrina*.

2. None of these enzymes were detected in the gastrodermis of starved animals. Acid phosphatase activity appears 5 minutes after feeding commences and is closely identified with formation of the food vacuoles. Maximum phosphatase activity is present in all vacuoles 24 to 48 hours after feeding; a slight reaction occurs in the cytoplasm of all gastrodermal cells at this time. Three days after feeding, acid phosphatase activity declines until one week after feeding, no activity is evident.

3. Aminopeptidase activity is present in the mesenchyme of starved animals where it is probably associated with "self-digestion" phenomena related to the nutritional state of the animal. In general, aminopeptidase activity parallels that of acid phosphatase. While intracellular localization of β -glucuronidase was not achieved, its activity in the digestive tract was observed to be similar to that of the other two enzymes studied.

4. The results obtained on a histochemical level are discussed in terms of current concepts of the mechanism of intracellular digestion.

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THE IN VITRO REACTION OF LIMULUS AMEBOCYTES TO BACTERIA ¹

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The horseshoe crab, *Limulus polyphemus*, an ancient marine arachnoid, reacts to infection by extensive intravascular clotting (Bang, 1956). Since this is primarily a cellular reaction, followed by extracellular gelation (Loeb, 1910), it was of particular interest to study the interaction of bacteria and the cellular components *in vitro*. The present paper reports (a) the maintenance of the normal granular discoid amebocytes in siliconized and unsiliconized glassware, (b) the destructive effect of the bacteria and bacterial toxin in first changing these cells to agranular and filiform extended cells and then destroying them, and (c) the ability of the granular discoid amebocytic preparations to suppress the development of nonpathogenic bacterial infections in the cultures.³

MATERIALS AND METHODS

Healthy, adult specimens of *Limulus*, 10 to 12 inches across the shell, mostly female, were obtained from the Marine Biological Laboratory Supply Department and kept in running sea water. No data were available concerning the previous history of infection among these animals.

Preparations without silicone—granular and agranular cells

Thirty to 50 ml. of blood were obtained by cardiac puncture and were placed directly into a series of unsiliconized 10-ml. screw-cap roller tissue culture tubes (2 ml. per tube) with routine sterile precautions. After some manipulations to obtain a uniformly thin layer of material over most of its inner curvature, the tube was placed in a rotating drum and kept at room temperature (20–25° C.). The cells were studied by direct microscopic examination of the tube and by removal of drops of the culture fluid⁴ with its suspended amebocytes. The latter were followed both by phase and direct transmitted light. A combination of 100 units each of penicillin and streptomycin was used in a few of the early experiments, but in most tubes no antibiotics were used. The effect of fluid changes was studied by replacing the media with various concentrations of adult

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² Predoctoral Research Fellow, National Institutes of Health.

³ Some of this material has been reported in abstracts: (Shirodkar, Bang and Warwick, 1958; Warwick and Bang, 1957).

⁴ Hereafter referred to as "medium" or as "serum."

Limulus serum. Wobach's modification of Giemsa's stain was used in order to determine the cytological details of the cells.

Cultures with silicone—cultures in siliconized glassware

In these experiments the culture tubes, syringes and needles were coated with silicone (G.E. SC-87 Dri-Film) and air-dried for 3–4 hours before they were thoroughly washed with the commercial washing powder "Gold Dust," rinsed several times with tap and then with distilled water and dry sterilized at 180° C. for 1½ hours. Large volumes of blood could not be withdrawn and transferred (2 ml. for each roller tube) without clot formation unless the apparatus was thus siliconized. Particularly careful manipulation was required immediately after explantation of the blood. The preparation was considered satisfactory only if a clear majority of the attached and suspended amebocytes was indistinguishable from those found *in vivo*, i.e. discoid, granular, (Lankester, 1884; Loeb, 1902), as studied by low power microscopy, for at least 48 hours following explantation.

Description of bacteria used

Limulus pathogen. In the summers of 1953, 1954, 1957 and 1958 organisms were isolated from the peripheral blood of sick adult animals by means of techniques described elsewhere, and the 1958 stock cultures were maintained by fortnightly transfers on ZoBell's sea water agar, enriched with peptone and ferric salts (Bang, 1956). They were stored between transfers at 4° C. On the basis of bacteriological tests⁵ the strain isolated in 1958 was identified as a *Vibrio* sp. It is gram-negative, short, thick, polarly monotrichous, motile and does not form spores. It is comma-shaped, particularly in old cultures. Thin capsules are occasionally present. The organism is facultatively anaerobic and has an optimum growth temperature of 22° C., complete cessation of growth occurring at 5° and 37° C. It produces β -hemolytic zones on horse blood agar; liquefies gelatin rapidly (1 day at 22° C.); shows presence of peroxidase and catalase; does not produce H₂S; gives a weak positive reaction with indole and reduces nitrate to nitrite. The bacterium is incapable of fixing nitrogen and shows neither fluorescence nor luminescence. It produces acid, but not gas, in the following carbohydrate media: glucose, galactose, maltose, trehalose, mannitol, starch, sorbitol (weak at 15 days), glycerol (weak) and salicin (weak). No acid is produced with arabinose (late positive, 15 days), xylose, rhamnose, sucrose, lactose, adonitol, dulcitol, inositol and ethyl alcohol.

A heat-stable toxin was obtained from this bacterium by the same method as in the previous work.

Bacterium #5 (Limulus nonpathogen). Pure cultures⁶ of this gram-negative peritrichous, motile rod were obtained at the beginning of the summer of 1958 from oysters.

Bacteriological studies showed no fermentation except in ethyl alcohol, which

⁵ These, as well as the tests on Bacterium #5, were kindly performed by Dr. H. Lautrop of the State Serum Institute, Copenhagen, Denmark, to whom we are grateful for the accompanying description.

⁶ These, as well as other cultures used here, were isolated by Mr. Stuart Krassner of our laboratory and kindly supplied to us.

suggested that it belongs in the *Alkaligenes* group. However, since it produces H_2S , it is tentatively designated *Alkaligenes*-like. When inoculated, even in large doses, into healthy, adult animals, these bacteria produced no demonstrable disease.

Other bacteria. Eight different bacteria were tested, seven of which had been isolated from oysters whilst one, a yellow chromogen (#19), was found as a laboratory contaminant. No attempt was made to fully identify any of them. They were distinguished one from the other principally on the basis of colony and cellular morphology. They were arbitrarily numbered 4, 10, 12, 14, 15, 17, 18, 19.

Antibacterial activity of cultures. The experiments designed to test the extent of antibacterial activity and to determine in which fraction(s) of blood it resided, consisted essentially of (a) setting up blood culture tubes, adding known amounts of bacteria, withdrawing aliquots from the mixtures at successive time intervals and plating these on agar to determine the drop in numbers of viable bacteria; (b) following the same procedure with tubes containing different fractions of the blood, such as serum with few granules, serum with many granules and with fresh, whole blood homogenates. The measures of antibacterial activity were (1) the largest initial inoculum following whose introduction into the system either no bacteria (or reduced numbers) were recoverable and (2) the length of time needed to achieve such reduction or to maintain inhibition at a constant level.

One ml. of sterile, artificial sea water (without $NaHCO_3$) was added to an agar slant containing a heavy 24–48 hour (room temperature) growth of the organism and mixed thoroughly. In the earlier experiments, the titers of such undiluted suspensions of the various bacteria were determined by making pour plates of serial $\log_{10}$ dilutions in sea water agar. The resulting values for viable bacteria were found to be consistently in the range of 10^9 to 10^{10} cells per 0.1 ml. We subsequently assumed these figures for freshly prepared suspensions. Inocula of 0.1 ml. of various dilutions were added to the experimental and control culture tubes; the tubes were kept at room temperature in the rotating drum, and samples were taken subsequently for culture by means of a platinum wire loop. Colonies were counted at 24 hours. In the series of experiments designed to yield information regarding the fraction(s) of blood containing the factor(s) responsible for the bacteria-reducing capacity of the cultures, fresh blood was aseptically explanted into unsiliconized Petri dishes which were then allowed to stand at room temperature for varying periods of time. A gel-like clot formed rapidly after explantation and was adherent to the bottom of the dish as described by Loeb (1920). Syneresis occurred and the supernatant serum contained a minimum number of free amebocyte cytoplasmic granules if drawn off within a few minutes after placing the blood in the dish. With stirring, this number increased steadily up to about two hours. Aliquots were removed from the supernate at intervals and the state of the granules was noted under the low and high dry powers of the light microscope, using blue light and no filter, with reference to shape, size, color, refractility and Brownian movement.

Three terms which will have particular connotations as used throughout this report may need clarification. A *normal granule* is one which appears like those seen in the intact cell. It has a uniform size; the shape is spherical to slightly ovoid; it appears green when seen through the ordinary light microscope, using blue light and no filter; has high refractility and shows a characteristic amplitude of Brownian movement when in serum. *Polymorphic granules* are considered

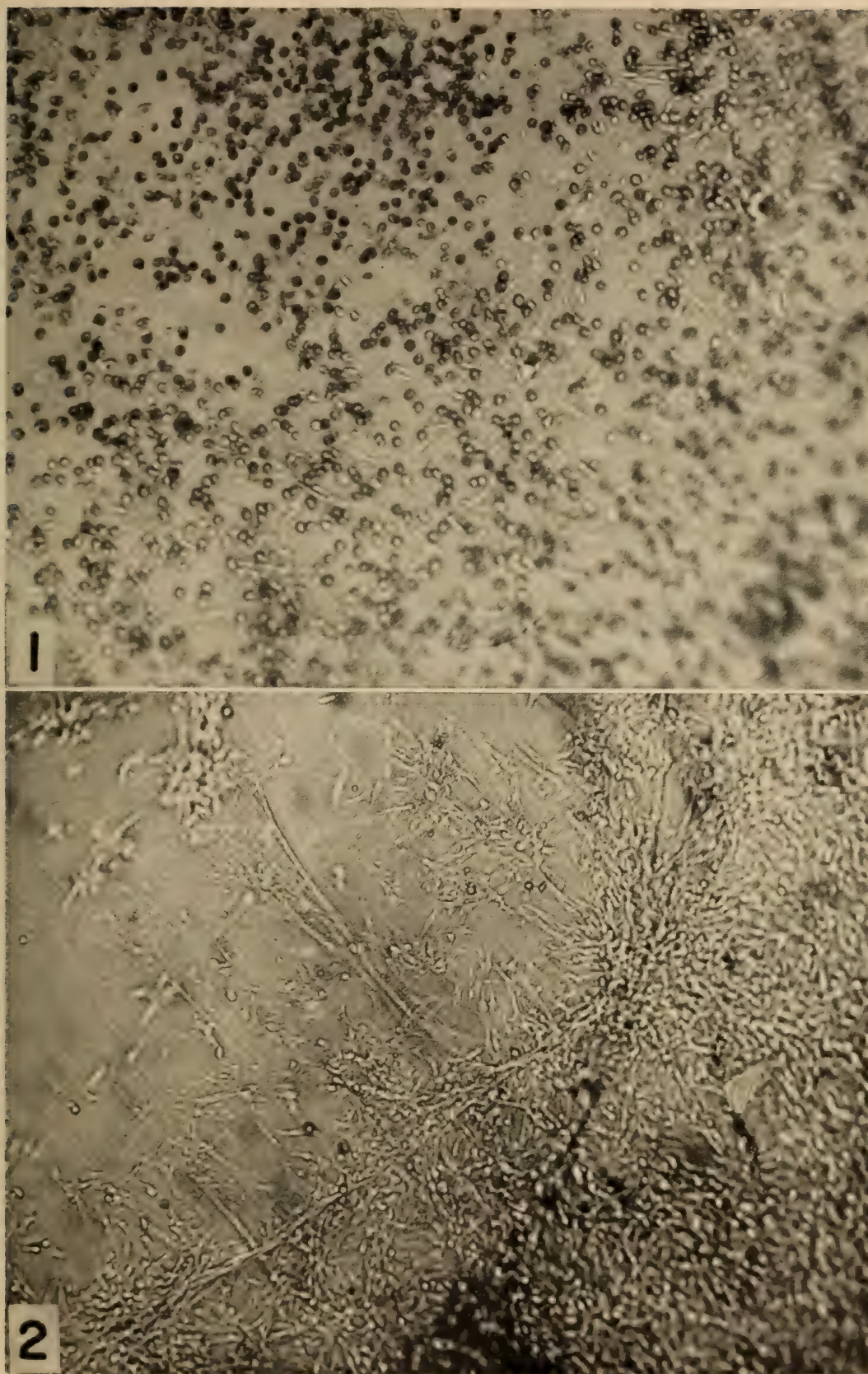
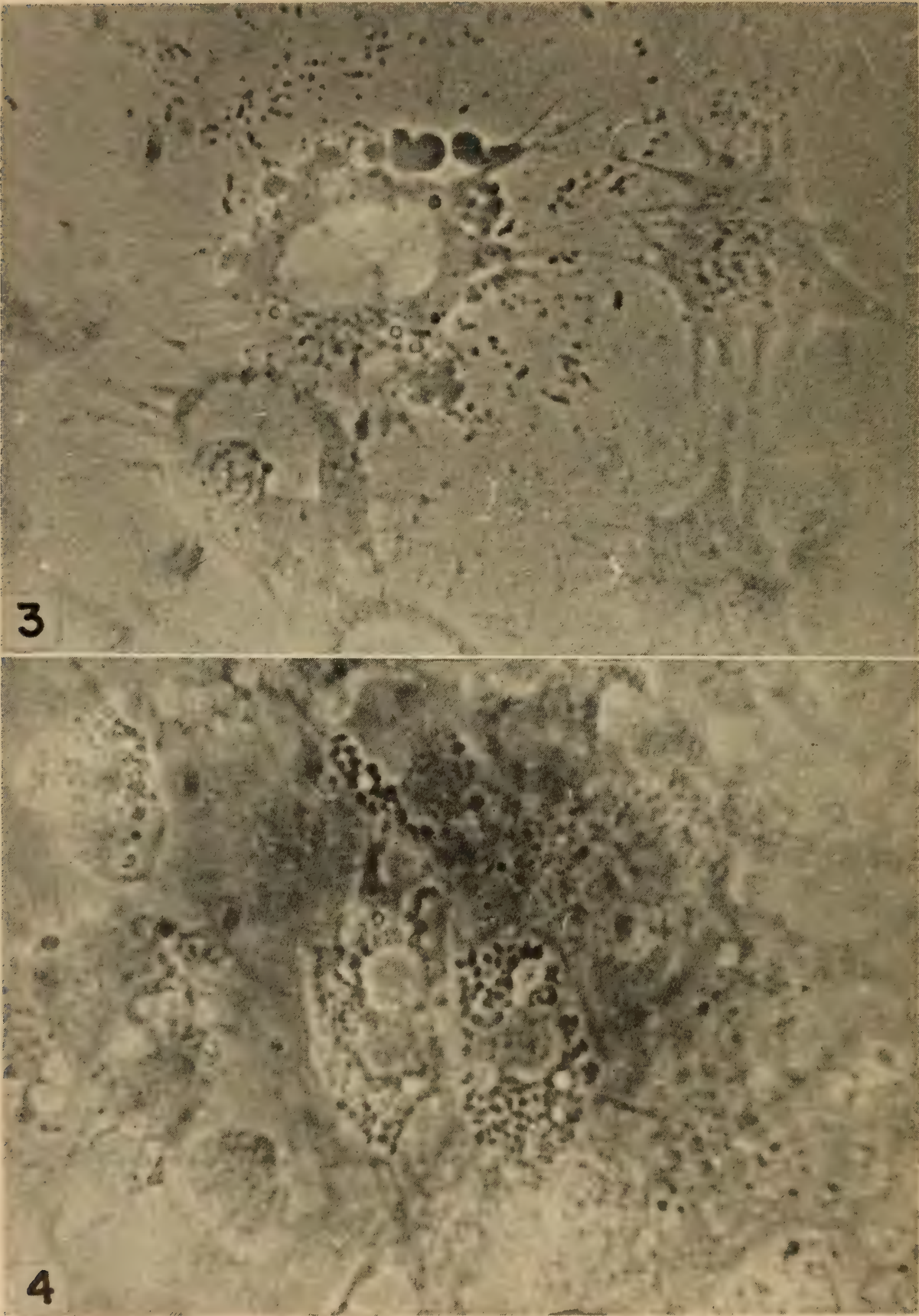


FIGURE 1. Amebocyte culture before addition of toxin. Note the discoid granular cells. Light areas represent some "hyaline," extended cells. (80 $\times$)

FIGURE 2. Same culture as in Figure 1, after addition of toxin. Amebocytes have lost granules and extended; many have apparently disintegrated.



FIGURES 3-4.

either degenerate or pathological, may be rod- or dumbbell-shaped and may be larger or smaller than the normal. *Translucent granules* are those which have lost their normal high refractility. They are usually a pale yellow-green.

RESULTS

In vitro survival of amebocytes

Variation in serum concentration. In the hope that a lower concentration of Limulus serum frequently renewed might lead to cellular growth, four different concentrations were tested: 10, 25, 50 and 100 per cent, some with glucose added. The cells remained viable for as long as 36 days at the 10 and 50 per cent concentrations, but were not followed as long at the 25 and 100 per cent concentrations. Although long-term observations were not made with the 100 per cent (undiluted) serum in these first experiments, it was noted that granular discoid amebocytes were commonly found in the undiluted serum but rarely or never at the 10 per cent concentration. In all cases, the number of these cells gradually diminished. In general, cell appearance varies from the thin, flattened, hyaline cells with no granules and sharp, extended pseudopod-like processes, to the extended but granular cells and, finally, the granular discoid amebocyte. Attempts to transfer the amebocytes by direct explantation, trypsinization, or the use of "Versene" failed. In none of the direct microscopic examinations, nor in the stained preparations of the cultures were mitotic figures seen, nor was there any conversion of the amebocyte tissue into an organized growth.

Undiluted Limulus serum was therefore used in the tests with the bacterial toxin, and partially degranulated cells were consistently obtained. It was found unnecessary to replace the nutrient medium (the serum) at any time even in experiments of several weeks.

Effect of siliconized glassware. By applying silicone to the apparatus, in the very first experiment and in subsequent good cultures, the amebocytes were maintained morphologically indistinguishable from those in the animal for as long as 30 days in the rotating drum at room temperature without replenishment of the culture medium; more will be said about this in the section on interaction with bacteria.

Effects of the Limulus pathogen and its toxin on the blood cultures. Since the generalized pathology of the disease, an intravascular clotting, can be reproduced by a heat-stable toxin, the effects of this toxin on amebocytes *in vitro* were studied. As is shown in Table I, the addition of various dilutions of toxin to cultures of amebocytes in normal clean glassware caused prompt changes in morphology, culminating in the apparent disintegration of the cells, and the formation of an extracellular gel. The cells lost their granules, extended long processes, and assumed bizarre shapes. They are shown in Figures 1 and 2.

In order to study these changes under higher power, a loopful of fresh undiluted bacterial (*Vibrio*) suspension was mixed with a loopful of intact amebocytes from

FIGURE 3. Changes in amebocytes caused by *Vibrio*. Note blebs and needle-like extensions of cytoplasm, large vacuoles and nucleus displacement. (Phase, 1400 ×)

FIGURE 4. Amebocytes without addition of *Vibrio*. The two central cells are intact. Some vacuole formation is present in neighboring cells. (Phase, 1400 ×)

siliconized roller tubes and examined on a slide by phase microscopy at $1400\times$. Controls were (a) cells with the sterile artificial sea water without NaHCO_3 , and (b) cells without such dilution.

A remarkable set of changes with the pathogen was observed. Destruction of the cell occurred within 5 minutes while the control cells, though definitely altered inasmuch as there was some pseudopod and vacuole formation, still contained

TABLE I
Effect of toxin on amebocytes in vitro

Dilution of toxin	Time following inoculation in hours		
	$\frac{1}{4}$	1	5
Undiluted	+	++	+++
1×10^{-1}	+	++	+++
1×10^{-2}	+	++	++
1×10^{-3}	+	++	++
1×10^{-4}	0	0	0
Control (sterile artificial sea water without NaHCO_3)	0	0	0

0 = Majority of amebocytes discoid, granular.

+

++ = Larger fraction of amebocytes extended and agranular; some disintegrated.

+++ = Majority of amebocytes extended and agranular or disintegrated.

normal granules and were not greatly changed. The changes in the cells with the *Vibrio* were:

(1) A rapid and uncoordinated protrusion and retraction of blebs of cytoplasm, reminiscent of pseudopod formation.

(2) The formation of multiple intracytoplasmic vacuoles and rapid loss of granules from the cell. The few granules which remained in the cell seemed enlarged and lost their normal refractility; later the extruded granules also enlarged and became translucent.

(3) Coalescence of the vacuoles into larger clear ones which occupied most of the cell and which often displaced the nucleus. Formation of sharp, needle-like extensions of the cytoplasm.

(4) Marked ballooning of the cell and cytolysis.

Figures 3 and 4 depict some of these changes.

The addition of the *Alkaligenes*-like bacteria produced no comparable changes in intact amebocytes during the same period of time, although here, too, some pseudopod and vacuole formation were observed.

An extracellular gel is present both during the normal clotting process and the pathological intravascular clotting (Bang, 1956). When the pathogen was added to the cultures, both siliconized and normal, a much heavier gel-like material invariably appeared in the medium and persisted as long as the cultures were observed. Microscopic examination showed no living cells, but a ropy network which enmeshed numbers of agglutinated swollen, polymorphic, translucent, spher-

ical or elongated bodies which probably were altered granules. Small discrete dark bodies were also frequently found with these.

Antibacterial action of the discoid granular cells. When the cultures of cells on siliconized glass were first set up no sterile precautions were taken, and yet the cultures remained free of bacteria. It was therefore of immediate interest to test the ability of these "intact" cells to eliminate bacteria. It was found that large numbers of the *Alkaligenes*-like bacterium were rapidly eliminated by these cells, whereas cultures in which the cells had changed to the extended agranular type (unsiliconized glassware) showed reduction and inhibition of the bacteria but that this was temporary. Tables II and III summarize typical experiments.

TABLE II

Effect of granular discoid amebocytes on Alkaligenes-like bacterium. Number of colonies recovered per loopful of fluid and tissue

Number of bacteria added*	Hours after addition of bacteria							
	1½	6	12	24	48	72	96	120
2.4×10^7	3	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0
					0	0	0	0
2.4×10^5	11	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0
					0	0	0	0
2.4×10^3	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
					0	0	0	0
Sterile artificial sea water without NaHCO_3	0	0	0	0	0	0	0	0
Nothing added	0	0	0	0	0	0	0	0

* Varying dilutions of a fresh suspension of bacteria were added to the cultures. The figures given were determined by the pour-plate method. Three tubes were used for each dilution but samples were withdrawn from only two up to 24 hours. This served as a check on a possible influence of the sampling procedure itself.

The apparent drop in bacterial count at 1½ hours might be explained by an estimated dilution of about 10^{-2} to 10^{-3} in the taking of small samples. However, at 6 hours tubes of the intact system showed no bacteria and from then on none were recovered from the granular cells. In cultures of extended cells (Table III) there seemed to be some inhibition but between the 24- to 72-hour sampling the bacterium reappeared and, by 72 hours, confluent growth was obtained. At the highest dilution (8.5×10^1 bacteria) no organisms were recovered at any time.

The experimental and control cultures were observed microscopically and loopfuls of fluid and tissue were examined on slides at 3½, 24 and 120 hours. There was no persistent gelation in the uninfected system or in those cultures which overcame the infection. The medium in these remained transparent and blue.

TABLE III

Effect of agranular extended amebocytes on Alkaligenes-like bacterium. Number of colonies recovered per loopful of fluid and tissue

Number of bacteria added*	Hours after addition of bacteria					
	2	6	12	24	48	72
8.5×10^5	42 33	4 9	0 1	1 0	80 Confluent 75	Confluent Confluent Confluent
8.5×10^3	10 5	1 0	0 0	0 1	50 100 3	Confluent Confluent Confluent
8.5×10^1	0 0	0 0	0 0	0 0	0 0 0	0 0 0
Sterile artificial sea water without NaHCO_3	0	0	0	0	0	0
Nothing added	0	0	0	0	0	0

* See footnote to Table II.

Cell changes were proportional to the number of bacteria inoculated. Cultures inoculated with 2.4×10^7 bacteria showed a majority of degranulated and flattened (but living) cells; those with 2.4×10^5 organisms had approximately equal proportions of degranulated and intact amebocytes, whilst the 2.4×10^3 tubes showed a great majority of intact cells, as did both controls. Furthermore, all cultures had apparently normal, free granules in the medium throughout the period of observation, with the greatest numbers at the highest and fewest at the lowest bacterial inoculum size and in the controls. Agglutinated clumps of dead bacteria were found in the experimental tubes.

The granular discoid cell cultures were ineffective in preventing the multiplication of the pathogenic *Vibrio*. Cultures inoculated with 10, 100, 1000 and 10,000 viable bacteria (direct colony count) showed destructive progressive infection, all

TABLE IV

Range of antibacterial activity of intact system against various unidentified marine bacteria

Bacterium stock number	Antibacterial activity
4	Good
10	
15 (slow grower)	
19	
14	Intermediate
18 (slow grower)	
12	None
17	

TABLE V

*Degenerative changes in amebocyte granules in serum (room temperature)
(unsiliconized glassware)*

Time (hours) elapsed subsequent to explantation of blood					
$\frac{1}{4}$	$\frac{1}{2}$	$4\frac{1}{2}$	$17\frac{1}{2}$	43	72
Few free in serum; all normal in shape, size, color and Brownian movement	Several in serum; all normal in shape, size, color and Brownian movement	Many in serum; all normal in shape, size, color and Brownian movement	Profuse in serum; most normal, some polymorphic, translucent, with altered Brownian movement	Profuse in serum; most polymorphic, translucent, some with red tinge and altered Brownian movement	Profuse in serum; all polymorphic, most with red tinge and altered Brownian movement

yielding confluent growth of bacteria 24 hours after inoculation. This experiment was repeated with the same result several times.

The effect of these "intact" siliconized cultures was then studied on several unidentified marine bacteria (Table IV). The activity was considered good if 1000 or more bacteria were eliminated. Thus the antibacterial activity was not limited to one strain of bacterium.

Eight different bacteria were used and three dilutions per bacterium, as described for the *Limulus* nonpathogen. The activity was studied at 2, 34 and 144 hours at room temperature.

An attempt was made to determine which of the components within the blood tissue culture system produced antibacterial activity. First, using unsiliconized glassware, the degeneration time of the cytoplasmic granules, once they had left the cell, was determined. Then antibacterial measurements were made with sera containing minimal and maximal numbers of granules as well as with whole blood homogenates at room temperature and at 0° C.

TABLE VI

*Antibacterial activity of whole blood homogenate on Alkaligenes-like bacterium at 0° C**

	Number of bacteria recovered per loopful		
	Hours after addition of bacteria		
	2	24	48
Fresh homogenate + 1×10^7 bacteria	Confluent	102‡	100
Control† + 1×10^7 bacteria	Confluent	Confluent	Confluent
Fresh homogenate + 1×10^5 bacteria	Confluent	16	12
Control + 1×10^5 bacteria	Confluent	Confluent	Confluent
Fresh homogenate + 1×10^3 bacteria	Confluent	9	7
Control + 1×10^3 bacteria	Confluent	50	Confluent

* After 48 hours at 0° C. all test and control tubes were returned to room temperature and so maintained for another 24 hours, when loopfuls were plated. Confluent growth was obtained in all cases.

† Control = 2 ml. of whole blood homogenate heated at 60° C. for $\frac{1}{2}$ hour.

‡ These figures represent the average of two experimental culture tubes.

Table V shows the degeneration time of the cytoplasmic granules outside of the cells to be between 17½ and 43 hours subsequent to explantation. This is interesting because of possible correlation with the 24- to 48-hour inhibition of bacterial growth seen in the extended cell system (Table III) and also because the discoid granular cell culture shows many perfectly normal granules 120 hours after addition of large numbers of the nonpathogenic *Bacterium* #5.

In tests for antibacterial activity of sera containing varying numbers of granules and of whole blood homogenates, equivocal results were obtained until the test for inactivation of the bacteria by a homogenate was done at 0° C. This has been found favorable for the measurement of the antibacterial activity of *Phascolosoma gouldii* blood (Bang and Krassner, 1958).

Table VI presents the results of this preliminary experiment.

DISCUSSION

The *in vitro* maintenance of *Limulus* amebocytes was described by Loeb (1920), who reported that hanging-drop cultures made from his "experimental amoebocyte tissue" contained well preserved cells showing ameboid movements for over a week, but eventually showed "degenerative changes." Cultures showed active amebocytic migrations but no multiplication. As early as 1905 and 1906, the same worker had observed the preserving effects, on the cells, of solutions of both dilute acid and alkali in isotonic NaCl (Loeb, 1905, 1907, 1910) and had found (Loeb, 1907, 1910, 1928) that the irreversible conversion of discoid, granular amebocytes to an extended, agranular, "hyalinized" state upon exposure to glass could be delayed by coating the surface with Vaseline or paraffin. The later publications of Loeb and associates (Loeb and Blanchard, 1922; Loeb, Bierman and Gilman, 1924; Loeb, Bierman and Genther, 1925; Loeb and Genther, 1927; Loeb and Genther, 1928) dealt primarily with the stimulating effect of acid and alkali on the cell migrations ("outgrowths").

It is believed that the 30-day maintenance period for amebocytes in an intact state in a siliconized system, as reported in the present paper, is the longest recorded, as is the 36-day period of viability for amebocytes in an extended hyaline state. Such prolonged maintenance of cells at 20°–25° C. without change of medium is in itself interesting. Our results are in agreement with those of Loeb in that (a) undiluted *Limulus* serum is the best natural medium for amebocytes (Loeb, 1920) and (b) that no cell multiplication occurs in such cultures (Loeb, 1920; Loeb, Bierman and Genther, 1925).

The siliconized system seems to be well suited for conducting the various experiments described. The observations regarding *in vitro* destructive effects of the toxin on the cells, progressive infection of the cultures, and cell changes produced by the pathogenic *Vibrio* itself *in vitro*, correlate well with effects previously noted (Bang, 1956) in the living animal, thus providing some indication as to the probable course of events in pathogenesis and strongly implicating the toxin in the production of pathology following inoculation of the organism. It should be noted here that Métalnikov (1927) demonstrated that injection of endotoxins of several different bacteria (one, even after boiling at 100° C.) were lethal to caterpillars of *Galleria melonella*.

The suggestion has been made (Bang, 1956) that amebocyte reactions to trauma

and infection are ancient and represent basic protective mechanisms. Gelation of the blood would serve to immobilize the bacteria. During the course of several experiments conducted in this investigation it was noted that, while the pathogenic *Vibrio* was motile in the siliconized cultures, the nonpathogen was rapidly agglutinated and killed. Thus, trauma could stimulate interactions between cells and fluid resulting in a clot preventing further ingress of bacteria, those already within being killed by defensive factors in the blood. The results reported in the present paper indicate that defense mechanisms against hemorrhage (gelation, clotting) and against infection (production of antibacterial substance or substances) are intimately linked in the interaction between cellular and fluid elements of *Limulus* blood. Loeb, as noted elsewhere (Bang, 1956), described two stages in clotting and pointed out (1902) that fibers of clotted blood are partly formed directly from amebocyte protoplasm. These observations were confirmed during the course of this work. The formation of an extracellular gel during the normal clotting process, during pathological intravascular clotting and following addition of the pathogenic *Vibrio* or its toxin to siliconized or unsiliconized cultures, offers further evidence regarding the intimacy between the protective mechanisms. The *in vitro* gel persisted after addition of the pathogen and contained pathologically altered intra- and extracellular granules in large numbers. This suggests that the granules may be involved in some way, and is supported by the finding that they (a) appear normal in the media of granular cultures which have eliminated the Alkaligenes-like bacteria, their numbers increasing with increased bacterial inoculum size; (b) appear altered not only in cultures infected with the pathogen but also in blood from infected animals (Bang, 1956); and (c) have a degeneration time, in serum at 20–25° C., of 17½–43 hours (Table V) which may correlate well with the 24–48 hour antibacterial activity seen in an extended cell system (Table III). Finally, pretreatment (parenteral) of *Limulus* with the *Vibrio* toxin renders the animal more susceptible to infection with the *Vibrio* as well as with certain nonpathogenic bacteria (unpublished observation), this being presumably due to a lack produced by the toxin, of normal amebocytes and, possibly, of granules.

It has been shown that the intact system alone can eliminate large numbers of marine bacteria of *more than one strain* (Table IV), which lends support to the thesis that the phenomenon is truly antibacterial and not simply a chance observation. The extended cell and whole blood homogenate systems merely exhibit different levels of temporary antibacterial activity. An explanation may be that the intact system provides a steady, pump-like activity on the part of the healthy granular amebocytes leading to the release of some thermolabile intracellular component(s) into the serum long enough to kill all of the bacteria. The fact that as few as 10–100 pathogenic organisms can multiply and overcome the system may well be ascribed to the inactivating action of their toxin on the hypothetical intracellular component(s) and, possibly, on the granules. Its destructive action on the amebocyte itself has already been noted.

SUMMARY

1. For effective *in vitro* study of the defense mechanisms against bacterial infection of *Limulus polyphemus* an unsiliconized and a siliconized culture system for blood cell maintenance were developed. The former contained viable but

partially degranulated amebocytes for at least 36 days, whilst the latter system kept the cells in an intact state resembling the *in vivo* for up to 30 days. Replenishment of the culture medium (undiluted *Limulus* serum) and use of antibiotics were found unnecessary. No cell multiplication was noted.

2. The reactions of the blood elements to certain nonpathogenic and pathogenic marine bacteria (and to their toxin) were studied. When a pathogenic *Vibrio* was added to the siliconized system of granular, discoid cells, persistent gelation of the medium regularly accompanied bacterial growth, the gel containing pathologically altered amebocytes and granules. While gelation is characteristic for shed normal *Limulus* blood, intact cultures—both uninfected as well as those which overcome the nonpathogenic *Alkaligenes*-like bacterium—often showed either absence or ephemeral presence of gel in the medium. Such cultures also contained normal granules and showed large clumps of agglutinated dead bacteria consistently, suggesting involvement of the gelation process in immobilization and, perhaps, killing of the invading organism. The culture systems were active against several strains of marine bacteria. Experiments with a nonpathogenic *Alkaligenes*-like bacterium showed that only the intact, granular cultures showed rapid (6 hours at 20–25° C.) and *permanent* elimination of large numbers (inoculum sizes up to several million), the unsiliconized extended cell and whole blood homogenate systems merely exhibiting different levels of evanescent activity.

3. The successful infection of the intact system, by very small numbers (10–100), of pathogenic *Vibrio* clearly established its *in vitro* pathogenicity. Its thermostable toxin caused prompt changes in the morphology of the intact amebocytes in culture, even at a 10^{-3} dilution, and a phase microscopic study of the action of the *Vibrio* itself on the cells revealed rapid cellular alterations culminating in cytolysis.

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THE SELECTIVE ACCUMULATION OF BLOOD PROTEINS BY THE OOCYTES OF SATURNIID MOTHS

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The growing oocyte of an insect accumulates proteins from the blood, rather than relying exclusively on the synthetic activities of the ovaries. Wigglesworth (1942) demonstrated in a number of blood-sucking insects that the modified hemoglobin which occurs in the oocytes is derived from the blood. More recently, an immunologically detected protein occurring in the blood of female saturniid moths has been found to leave the blood during the maturation of the ovaries and to accumulate in the oocytes (Telfer, 1954). That such a process might be entailed in the growth of oocytes other than those of insects is suggested by the antigenic and physical similarities between oocyte and serum proteins of chickens (Schechtman, 1947; Nace, 1953; Schjeide and Urist, 1956) and of frogs (Cooper, 1948; Flickinger and Rounds, 1956).

A variety of other animal cells can accumulate soluble proteins from their environment and, of these, one of the most thoroughly analyzed has been the amoeba (Holter, 1959). The mechanism of protein uptake in the amoeba has been proposed to include two essential features: adsorption of the protein on the surface of the cell (Marshall, Schumaker and Brandt, 1959), and a subsequent infolding of the cell surface to form vacuoles (Mast and Doyle, 1934; Chapman-Andresen and Prescott, 1956). The latter process was first described by Lewis (1931) who observed mammalian cells in tissue culture ingesting their culture medium.

An analysis of protein accumulation by the moth oocyte has been undertaken. Included in this analysis is an attempt to determine whether the mechanism involved is comparable to that proposed for the amoeba. Since, unlike the amoeba, the moth oocyte cannot be observed microscopically during the process of protein accumulation, studies of the mechanism must in this case be based on less direct methods. Evidence is presented here that most proteins in the blood of the moth enter the oocyte to some extent but that, from a quantitative point of view, the mechanism is highly selective. The bearing of this and other evidence on the nature of the protein accumulating mechanism will be considered in later papers.

MATERIALS AND METHODS

The capacities of seven proteins to enter the oocytes from the blood of the *Polyphemus* moth were assayed by means of a quantitative application of Oudin's antiserum-agar technique. Two of the proteins occur in the blood of the *Cecropia* moth and normally accumulate in the oocyte of that species. This has already been demonstrated for one protein, which will be referred to as the "female protein,"

¹ This work was performed in part while the author was a Summer Fellow of the Lalor Foundation, and in part with the support of Phi Beta Psi Sorority.

and it is demonstrated here for a second protein found in the blood. The five additional proteins were derived from the blood or oocytes of animals other than insects.

1. *Injection of foreign proteins*

The protein preparations were introduced into diapausing female pupae of the *Polyphemus* moth which had previously been chilled for several months at 6° C. in order to activate the endocrine mechanism responsible for stimulating their transformation into moths (Williams, 1956). The *Cecropia* proteins were introduced into pupae by the transfusion technique previously described (Telfer, 1954). Proteins of non-insectan origins were injected into the haemocoel from a tuberculin syringe through a No. 28 hypodermic needle. The volume of the protein solution, in milliliters, was equal to one-twentieth of the weight of the pupa in grams, in the case of the non-insectan proteins, and to one-fifth of the weight of the pupa in the case of *Cecropia* blood. In order to avoid melanin formation which can occur in the blood in response to surgical shock, approximately 0.1 mg. of phenylthiourea was included in the solution introduced into each animal.

2. *Preparation of the blood and oocyte fluid of Polyphemus moths*

Oocyte growth occurs in *Polyphemus* primarily during the final half of the period of transformation of the pupa into the moth. The injected animals were bled at the end of this period. The blood was collected in test tubes and stored under mineral oil at -20° C. The ovaries were thoroughly washed with 0.15 M NaCl, blotted and placed in a mortar. Most of the oocytes were shaken free of the ovarioles during the washing procedure, and the chorions of these oocytes were broken by pressure applied with a pestle. The yolky contents that were thus squeezed out of the chorions were centrifuged at $20,000 \times g$ for thirty minutes at 0° C. After centrifugation, a clear, yellow, aqueous layer, which will be referred to as oocyte fluid, lay between a sediment and a floating, particulate layer. The clarified oocyte fluid occupied approximately three-quarters of the volume of the centrifuged oocyte contents. It was analyzed, along with the blood, for its content of the protein in question. Melanin formation was prevented in both blood and oocyte fluid by the addition of crystalline phenylthiourea.

3. *Preparation of the protein solutions for injection into Polyphemus pupae*

Commercial preparations of chicken ovalbumin, bovine gamma globulin and bovine serum albumin (all Pentex) were dissolved in 0.15 M NaCl at neutral pH. A 2% solution of a protein preparation was used for injection into pupae.

In addition, extracts of chicken's egg yolk and of lobster oocytes were studied. The chicken yolk extracts were prepared by washing several yolks free of the white and then breaking them in several times their volume of 0.15 M NaCl. The mixture was dialyzed against saline solution and finally centrifuged at $20,000 \times g$. The final preparation, which was an opalescent yellow solution, was injected into pupae without further dilution. Judging from the ionic strength of the extraction medium, this preparation should have included the protein complexes, lipovitellenin and livetin (Fevold, 1951).

Lobster oocytes were obtained from ovaries dissected from living lobsters (*Homarus americanus*). Developing lobster oocytes, after passing through yellow and pale green phases, finally attain a dark green color. The oocytes extracted here were at least 2 mm. in diameter and were dark green. They were loosened from the ovarian wall by agitation in 0.15 *M* NaCl. After being washed several times, the oocytes were disintegrated in a Virtis homogenizer in several times their volume of saline solution. The extract, after dialysis against saline and centrifugation at $20,000 \times g$, was a clear, dark green solution. The green color of lobster oocytes is due to ovoverdin, the carotenoid-protein complex studied by Stern and Salomon (1937).

Finally, two proteins of *Cecropia* blood were studied. These were initially discovered by means of immunological techniques and were designated as antigens 3 and 7 (Telfer and Williams, 1953). *Polyphemus* pupae were transfused with whole blood of *Cecropia* female pupae, which contains both of these proteins.

Most of the non-insectan protein solutions and extracts studied were toxic to some of the injected pupae and in these cases death occurred within three days after the injection. Such pupae never initiated their development into moths. The surviving animals, on the other hand, transformed into moths in the usual three-week period of time. Their ovaries produced eggs which appeared to be normal in size and structure, although sometimes reduced in number. Three preparations, bovine fibrinogen, phosvitin from hen's eggs and hemocyanin from lobster blood, were toxic to all pupae injected, and efforts to study the uptake of these proteins by the oocytes were therefore abandoned.

4. Immunological procedures

Antisera were obtained from rabbits. The reaction between a protein and its antibodies was studied by means of the antiserum-agar technique developed by Oudin (1948) and previously applied to the study of insect blood and oocyte proteins (Telfer and Williams, 1953; Telfer, 1954). A rabbit antiserum was solidified with agar in glass tubes (3 mm. i.d.) and was then overlaid with the blood, oocyte fluid or other sample whose content of the homologous protein was being tested. The antiserum was diluted to an appropriate level with 0.15 *M* NaCl buffered at pH 7.0. The rate of advance of the zone of antigen-antibody precipitation through the antiserum-agar (distance travelled/square root of time) was used as an index to the concentration of the protein serving as the antigen. The rate of advance of a zone of precipitation was converted to the relative concentration of the corresponding protein in the antigen layer by reference to a standard curve. Standard curves were constructed for each protein studied by determining the rate of advance of the zone of precipitation produced by a series of dilutions of a solution of the protein.

Precautions were taken which were previously recommended (Preer and Telfer, 1957) for reducing the effects of substances not involved in the reaction on the rate of advance of the zone of precipitation: in order to eliminate errors due to viscosity differences, the composition of the antiserum-agar was kept constant in any set of tests; and all tests were set up so that convection mixing occurred in the antigen layer (tubes vertical, antigen layer above the antiserum-agar, and the density of the antigen layer greater than that due to the diffusible components of the antiserum-agar).

In addition, it was demonstrated that samples of oocyte fluid, blood and 0.15 *M* NaCl, which were known to contain equal concentrations of an antigen, produced zones of precipitation with the same rates of advance (Table I). Therefore, with the exception of possible special cases, the Oudin test as applied here provided a valid criterion for comparing antigen concentrations in the blood and oocyte fluid of the moth.

5. Antigenic characteristics of the foreign proteins

All of the preparations of non-insectan proteins, except the lobster oocyte extract, on reacting in Oudin tubes with a 1:3 dilution of their homologous antisera produced a single dense zone of precipitation and, in several cases, additional weak zones. In order to achieve maximum sensitivity for the detection of

TABLE I

Comparison of the oocyte fluid and blood of Polyphemus moths as solvents for ovalbumin in antiserum-agar tests. Antigen solutions were layered over a 1:3 dilution of anti-ovalbumin serum

Dilution of a 2% ovalbumin solution in antigen layer	Rate of advance of the zone of precipitation when ovalbumin was diluted with		
	Oocyte fluid	Blood	0.15 <i>M</i> NaCl*
	$\times 10^{-3}$ cm.-sec. ⁻¹	$\times 10^{-3}$ cm.-sec. ⁻¹	$\times 10^{-3}$ cm.-sec. ⁻¹
1:1	3.2	3.2	3.3
1:2	2.9	3.1	—
1:4	2.7	2.8	2.8
1:8	2.5	2.5	—
1:16	2.2	2.2	2.2
1:32	1.9	1.9	—
1:64	1.6	1.6	1.7
1:128	1.3	1.3	—
1:256	1.1	1.1	1.1

* Since 0.15 *M* NaCl is less dense than the 1:3 dilution of antiserum, the Oudin tubes were placed horizontally in this case in order to assure convection in the antigen layer.

these antigens in Polyphemus blood and oocyte fluid, the antisera were diluted as much as was consistent with a clear visualization of the densest zone of precipitation. As a result, the weaker zones were generally not visible and information was thus obtained concerning only the antigen producing the densest zone. Two dense zones were visible in the reaction between the lobster oocyte extract and its homologous antiserum. Since the densities and rates of advance of these two zones were extremely similar, they were treated as a single zone and the rate of advance was determined only for the faster of the pair.

RESULTS

1. Demonstration of an oocyte antigen which is indistinguishable from a carotenoid protein of the blood

Evidence that *Cecropia* oocytes contain a protein which is antigenically similar to the female protein of the blood was described earlier (Telfer, 1954). Ob-

servations are described here which indicate that, in addition to the female protein, one of the several carotenoid proteins of the blood also has an antigenic counterpart in the oocyte.

Adult antiserum *d*, a pooled antiserum obtained from three rabbits which had been immunized with an extract of *Cecropia* female moths, produced two zones of dense precipitation when overlaid in Oudin tubes with either the oocyte fluid or the blood of female moths. One of these zones was not ordinarily produced by male blood and was thus identifiable as resulting from the precipitation of antigen 7, the female protein. The second, more slowly moving zone was due to precipitation of a substance previously designated as antigen 3 and characterized as a carotenoid protein. This identity was established in mutual dilution tests (Telfer and

TABLE II

The rates of advance of the two major zones of precipitation appearing when antiserum-agar containing adult anti-serum d (1:3) was overlaid with graded mixtures of oocyte fluid and the blood of female pupae

Composition of the antigen layer ($\frac{\% \text{ blood}}{\% \text{ oocyte fluid}}$)	Rate of advance of the zone of precipitation due to	
	Female protein	Carotenoid protein
$\frac{100}{0}$	$2.01 \times 10^{-3} \text{ cm.} \cdot \text{sec.}^{-1}$	$1.26 \times 10^{-3} \text{ cm.} \cdot \text{sec.}^{-1}$
$\frac{87.5}{12.5}$	2.04	1.29
$\frac{75}{25}$	2.11	1.29
$\frac{50}{50}$	2.17	1.35
$\frac{25}{75}$	2.23	1.41
$\frac{12.5}{87.5}$	2.23	1.44
$\frac{0}{100}$	2.28	1.49

Williams, 1953) in which aliquots of a sample of pupal blood were layered over graded mixtures of adult antiserum *d* and adult antiserum *a*, the antiserum whose reaction initially distinguished the carotenoid protein.

That the two zones of dense precipitation produced by oocyte fluid, on the one hand, and female blood, on the other, were due to related antigens was indicated by the results of mutual dilution tests. When graded mixtures of pupal female blood and oocyte fluid were layered over adult antiserum *d*, the two zones of dense precipitation behaved as shown in Table II. The rate of advance of each zone shifted gradually from that in the tube containing only blood to that containing only oocyte fluid. There was no detectable splitting, fusion, or crossing of the two zones. With regard to the precipitations visible in these tests, one can

therefore conclude that, as in the case of the female protein zone, the antibodies which form a single zone of precipitation with a carotenoid protein of the blood also form a single zone of precipitation with an antigen diffusing from the oocyte fluid.

Absorption tests provided additional evidence that the oocyte fluid contains an antigen similar to the carotenoid protein of the blood. Immunization of four rabbits with oocyte fluid and of three rabbits with female pupal blood led in every case to the production of antibodies which, according to mutual dilution tests with adult antiserum *d*, precipitated both the female and carotenoid proteins of the blood. All of these antisera, on absorption with an equal volume of a 1:10 dilution of

TABLE III
Absorption of antisera against Cecropia oocyte fluid with the blood of female pupae

Antiserum (final dilution 1:3 in all cases)	Zones of precipitation produced when oocyte fluid was layered over agar containing			
	Unabsorbed antiserum		Antiserum absorbed with blood of female pupae	
	Intensity of precipitation	Rate of advance	Intensity of precipitation	Rate of advance
Oocyte fluid <i>a</i>		$\times 10^{-3}$ cm.-sec. ⁻¹		$\times 10^{-3}$ cm.-sec. ⁻¹
	Weak	2.17	Weak	2.20
	Strong*	2.04	Weak	1.24
	Strong**	1.16	Weak	0.81
Oocyte fluid <i>b</i>	Weak	2.26	Weak	2.19
	Strong*	1.59	Weak	1.55
	Strong**	1.36		
Oocyte fluid <i>c</i>	Weak	2.17	Weak	2.31
	Strong*	2.10		
	Weak	1.36		
	Strong**	0.99		
Oocyte fluid <i>d</i>	Weak	2.30	Weak	2.39
	Strong*	1.52		
	Strong**	1.25		

* Zone of precipitation of the female protein (antigen 7).

** Zone of precipitation of the carotenoid protein (antigen 3).

oocyte fluid, lost their capacity to precipitate either blood or oocyte antigens in Oudin tests. Thus the oocyte fluid contains antigenic counterparts of all the substances, including the carotenoid and female proteins, detected in the blood with these four antisera.

Absorption of the antisera with female blood led to different results. As would be anticipated, the absorption of female blood antisera with pupal female blood removed all of the antibodies visibly precipitating either blood or oocyte antigens in Oudin tests. However, antisera against oocyte fluid, after absorption with female blood, retained the capacity to form from one to three zones of weak precipitation when overlaid by oocyte fluid (Table III).

Absorption tests thus indicated that the oocyte contains, in addition to the blood antigens precipitated by these antisera, at least three antigens which have not been detected in pupal female blood. The fastest zone produced when oocyte fluid reacted with the blood-absorbed antisera had, in each case, an intensity and rate of advance similar to the fastest zone produced by the unabsorbed antiserum. Oocyte antisera *a* and *b* produced additional zones whose failure to appear in the reactions with unabsorbed antisera was probably due to masking by the faster and denser zone of female protein precipitation (Table III). An alternative possibility of interest to the present problem is that at least one of these zones could have been due to residual antibodies against the oocyte's carotenoid protein—antibodies whose failure to react with the carotenoid protein of the blood would thus indicate an antigenic difference between the carotenoid proteins of the blood and of the oocyte. If this were the case, however, the amount of antibody involved was small in comparison with the total amount of carotenoid protein antibodies present in unabsorbed antiserum. The weak zones produced by a 1:3 dilution of the unabsorbed antiserum were comparable in intensity to the carotenoid protein precipitation in 1:30 dilutions of unabsorbed antiserum. Thus, at least 90% and possibly all of the antibodies precipitated by the carotenoid protein of the oocyte were also precipitated by the carotenoid protein of the blood.

Mutual dilution and absorption tests therefore led to the conclusion that the oocyte fluid contains a substance which is antigenically similar to and probably identical with antigen 3, a carotenoid protein of the blood.

2. *The relative concentrations of the carotenoid and female proteins in the oocyte fluid and the blood of Cecropia moths*

The concentrations of the carotenoid and female proteins in both the oocyte fluid and the blood were estimated from the rates of advance of their respective zones of precipitation. When the oocyte fluid and blood collected simultaneously from the same moth were layered over a 1:3 dilution of oocyte antiserum *c* in Oudin tubes, the zones of carotenoid protein precipitation advanced through the antiserum-agar at approximately equal rates. The zone of female protein precipitation advanced through the agar considerably faster from the oocyte fluid than from the blood, a result which is in accord with earlier observations on the female protein zone and which can be attributed to a disparity in concentration of this protein between the blood and the oocyte fluid (Telfer, 1954). Reference of the rates of advance of these zones of precipitation to standard curves yielded the relative antigen concentrations summarized in Table IV. This interpretation of the rates of advance led to the conclusion that the carotenoid protein is approximately equally concentrated in the oocyte fluid and blood of the moths, while the female protein is, on the average, twenty-eight times more concentrated in the oocyte fluid than in the blood.

3. *The accumulation of Cecropia blood proteins by Polyphemus oocytes*

Evidence that the carotenoid protein of the oocyte has its origin in the blood, rather than being synthesized in the ovary, was obtained from experiments in which the blood of *Cecropia* pupae was injected into female *Polyphemus* pupae.

TABLE IV

Relative concentrations of the female and carotenoid proteins in the oocyte fluid and in the blood of female Cecropia moths

Animal no.	Relative concentration of female protein		Relative concentration of carotenoid protein		Concentration ratio $\left(\frac{\text{oocyte fluid}}{\text{blood}}\right)$	
	Oocyte fluid	Blood	Oocyte fluid	Blood	Fem. prot.	Carot. prot.
1	1.15	0.03	1.00	0.77	38	1.3
2	0.93	0.03	0.85	0.81	38	1.1
3	1.05	0.06	1.45	1.25	18	1.2
4	0.90	0.04	0.81	0.81	21	1.0
5	0.76	0.03	—	—	29	—
6	1.15	0.04	0.66	1.20	31	1.8
7	1.05	0.06	1.12	1.20	19	1.9
8	1.05	0.03	0.71	1.35	37	0.5
Mean					28	1.1

When these animals had transformed into moths, their oocyte fluid and blood were obtained and were layered over adult antiserum *d* which had previously been absorbed with the blood of Polyphemus female pupae. After absorption in this manner, the antiserum retained its capacity to produce its two major zones of precipitation when overlaid with female Cecropia pupal blood, although, due to the removal of some antibodies, the zones were somewhat reduced in intensity. Thus Polyphemus blood contains substances which are antigenically similar to both the female and carotenoid proteins of Cecropia but which are distinctive enough so that they are unable to precipitate all of the antibodies against the Cecropia proteins.

Studies of the amount of the two Cecropia proteins in Polyphemus blood or oocyte fluid require that the rate of advance of the Cecropia protein's zone of

TABLE V

The relative concentrations of Cecropia's carotenoid and female proteins in the oocyte fluid and blood of Polyphemus female moths

Animal no.	Relative concentration of female protein		Relative concentration of carotenoid protein		Concentration ratio $\left(\frac{\text{oocyte fluid}}{\text{blood}}\right)$	
	Oocyte fluid	Blood	Oocyte fluid	Blood	Fem. prot.	Carot. prot.
1	1.1	0.05	1.0	0.7	22	1.4
2	1.1	0.05	0.5	0.6	22	1.0
3	1.5	0.05	0.8	0.7	30	1.1
4	0.7	0.05	0.8	0.4	14	2.0
5	1.0	0.05	0.4	0.8	20	0.5
Mean					21	1.2

precipitations be unaffected by the Polyphemus proteins present in the antigen layer. This requirement was found to be fulfilled: the blood of Polyphemus female pupae was indistinguishable from 0.15 *M* NaCl as a diluent for the two Cecropia antigens reacting with the Polyphemus-absorbed antiserum. Thus, the Oudin test could be used to indicate not only the presence, but also the concentrations of these two Cecropia proteins in Polyphemus moths.

Both the blood and the oocyte fluid of Polyphemus moths which had been transfused with Cecropia blood produced the two characteristic zones of dense precipitation in Oudin tests with the Polyphemus-absorbed adult antiserum *d*. The carotenoid and female proteins of Cecropia therefore found their way into the Polyphemus oocytes from the blood, an observation which has already been reported for the female protein.

When the rates of advance of the two zones of precipitation were converted to the relative concentrations of their respective antigens, the results recorded in Table V were obtained. The distribution of Cecropia's female and carotenoid proteins between the blood and oocytes of Polyphemus was approximately the same as their normal distribution in Cecropia (Table IV). This result suggests that neither the carotenoid protein nor the female protein of the oocyte fluid is synthesized in appreciable amounts in the ovary, but that both are derived primarily, and perhaps exclusively, from the blood.

4. *Similarities between other antigens of the oocyte fluid and blood*

The demonstration that the carotenoid and female proteins of the oocyte fluid are derived primarily from the blood raises the question as to whether other blood proteins participate in oocyte formation. The reactions of Cecropia oocyte fluid with adult antiserum *a* and with larval antiserum *c* suggest that this is the case. These two antisera have been shown to react with a minimum of six different Cecropia blood proteins, one of which is the carotenoid protein studied here (Telfer and Williams, 1953). In Oudin tests, each antiserum produced up to seven zones of precipitation when overlaid by Cecropia oocyte fluid. After absorption with equal volumes of a 1:10 dilution of oocyte fluid, none of the antisera was able to produce zones of precipitation when overlaid with Cecropia pupal blood. Substances which are antigenically similar to at least five blood proteins, in addition to the female and carotenoid proteins, are therefore present in the oocyte fluid. This result is consistent with the possibility that many, or even all, of the proteins normally present in the moth's blood enter the oocyte fluid to some extent, although synthesis in the ovary has not yet been ruled out in all these cases.

5. *The accumulation of non-insectan proteins by Polyphemus oocytes*

Less equivocal evidence that the oocytes can accumulate blood proteins other than the female and carotenoid proteins was obtained from experiments in which five antigens which are completely foreign to the moth were injected into the blood. Neither the blood nor the oocyte fluid of uninjected moths produced zones of precipitation in Oudin tests with antisera against the five non-insectan antigens studied. Therefore, the appearance of the antigenic activity of the injected protein preparations in the oocyte fluid could be interpreted as due to accumulation from the blood, rather than to synthesis in the ovary.

Of the five antigens injected into *Polyphemus* pupae, four were detectable in Oudin tests in both the blood and the oocyte fluid of the subsequently emerging moths. Only the major antigenic component of the chicken's yolk extract was undetectable in the oocyte fluid. The conclusion thus seems warranted that most proteins present in the blood are accumulated to some extent by the oocyte.

The relative concentrations of the foreign antigens in the oocyte fluid and blood were estimated from the rates of advance of their zones of precipitation (Table VI). The concentration of *Cecropia*'s female protein in *Polyphemus* oocyte fluid, relative to its concentration in the blood at the conclusion of egg formation, was approximately 200 times greater than that of the non-insectan proteins. Thus, while

TABLE VI
*Relative concentrations of foreign antigens in the oocyte fluid
and blood of Polyphemus moths*

Antigen	Concentration ratio (oocyte fluid/blood)		Number of cases
	Mean	Range	
<i>Cecropia</i> female protein	21	14-30	5
<i>Cecropia</i> carotenoid protein	1.2	0.5-2.0	5
Bovine gamma globulin	0.19	0.08-0.60	6
Bovine serum albumin	0.07	0.06-0.11	7
Ovalbumin	0.07	0.04-0.13	4
Lobster oocyte antigen (probably ovoverdin)	0.10	0.06-0.16	7
Chicken yolk antigen (probably lipovitellenin)	Undetectable in oocyte fluid	0.03-0.20	4

most proteins in the blood appear to enter the oocyte to some extent, different proteins vary greatly in their capacity to do so.

6. *Localization of the blood proteins within the oocyte*

The oocyte fluid analyzed in the Oudin tests described here consisted primarily of a solution derived from the breakdown of the yolk bodies. When whole oocytes were centrifuged at $20,000 \times g$, the strata which were formed within the chorion appeared different from the strata produced by the centrifugation of crushed oocytes. While a centripetal layer presumably consisting of fatty materials was formed in both cases, a non-particulate intermediate layer comparable to the clarified yellow fluid of crushed oocytes was not produced in whole oocytes. More than 90% of the volume of centrifuged whole oocytes was occupied by a sediment which, on dissection, was seen to consist of yellow spheres whose diameter ranged up to greater than 20 microns. Since more than three-quarters of the volume of crushed oocytes forms a clear, yellow liquid on centrifugation, it appears that the yellow spheres of undamaged oocytes are liquified when the eggs are crushed with a mortar and pestle. The nature of these particles, which we presume to be the yolk spheres of the cytologists (Wilson, 1925), will be considered in more detail in a later paper.

While the oocyte fluid presumably contained the non-particulate ooplasm and

fractions of any less voluminous particles which may have been damaged when the yolk was being expressed from the chorion, its primary constituent must have been lysed or liquified yolk spheres. The possibility thus appears that the proteins derived from the blood may be localized in the yolk spheres of the oocyte. This proposal is confirmed by the fact that the yolk spheres are colored bright yellow, for at least one of the proteins found here to be accumulated by the oocyte is a carotenoid protein which contributes to the yellow color of the blood.

DISCUSSION

The oocytes of saturniid moths have now been demonstrated to accumulate the female protein and a carotenoid protein from the blood. Antigenic similarities between the oocyte fluid and the blood suggest that other proteins which normally occur in the insect's blood may also be accumulated by the oocyte, although in these cases synthesis within the ovary has not been excluded. In addition, four proteins of non-insectan origin, after injection into the blood, have been found in the oocyte. Thus far, the only protein which has not been detected in the oocyte after injection into the blood is one derived from chicken yolk extract. Whether some feature of the uptake mechanism prevents the accumulation of this molecule or whether, in the course of accumulation, its antigenic properties are destroyed has not been determined. In experiments similar to some of those described here, Knight and Schechtman (1954) demonstrated that foreign proteins pass from the serum to the ovum of chickens.

There are large differences in the quantitative distribution of the various proteins between the oocyte fluid and blood. The data (Table VI) suggest that the mechanism of accumulation is uniquely adapted to the removal of particular proteins from the blood, and that others, such as the non-insectan proteins injected into the pupa may, in effect, be carried into the oocyte as contaminants. This interpretation presumes that the oocyte fluid: blood concentration ratios measured here are determined primarily by the capacity of the protein in question to penetrate the various cellular layers of the ovary and the surface of the oocyte. However, other events occurring within the injected insect could also affect this ratio and these must be considered in any attempt to apply the ratio of concentrations to an analysis of the protein accumulating mechanism.

Structural alterations which occurred in a protein molecule during or after its entry into the oocyte could, by affecting the rate of advance of the corresponding zone of precipitation, lead to gross errors in determinations of its distribution ratio. Such alterations could act in two ways: by altering the protein's antigenic properties and thus its capacity to combine with antibodies, or by altering its diffusion coefficient. A significant alteration in antigenic properties during the accumulation of the carotenoid and female proteins can be ruled out, since as is shown here, the blood and oocyte representatives of these proteins are extremely similar, and are possibly identical in their capacity to precipitate antibodies. It is also unlikely that the antigenic activities of the non-insectan proteins studied were grossly modified during their accumulation by the oocytes. In these cases, the zones of precipitation produced by the blood and oocyte fluid of the injected animals appeared to be equally dense and thus to entail the precipitation of comparable amounts of antibody. Antigenic changes which might not have been detectable by

this criterion could not account for the 200-fold differences observed in the oocyte fluid: blood concentration ratios of the proteins studied here. In a later paper, data will be presented which indicate that a change in diffusion coefficient does not occur during the transfer of either the female or carotenoid protein from the blood to the oocyte. It is therefore improbable that the distribution ratios measured for the seven proteins under consideration were subject to errors resulting from molecular alterations associated with the process of accumulation. Knight and Schechtman (1954) found that, in the chicken also, foreign proteins injected into the serum were not detectably altered by transmission to the ovum.

A protein's distribution between oocyte fluid and blood could be affected by secondary processes which are unrelated to the transfer mechanism: processes such as adsorption on an insoluble structure in the oocyte, or a selective destruction in the blood or other tissues after accumulation by the oocyte has been completed. While such processes may occur to some extent, the following observations indicate that the difference between the oocyte fluid: blood concentration ratios of the female and carotenoid proteins does in fact reflect a difference in their capacity to penetrate the oocyte. During the period of maximal oocyte growth, the concentration of female protein in the blood decreases 80%, while that of the carotenoid protein decreases only 20–25% (Telfer and Rutberg, 1960)—a difference which is readily accounted for by the female protein's being removed from the blood at a faster rate than the carotenoid protein. In addition, selective accumulation of the female protein can explain the effects of ovariectomy on the blood of female moths (Telfer, 1954). The electrophoretic pattern of the blood of moths which had been ovariectomized as pupae was similar to that of the blood of normal moths except for the fact that the moths which had not been allowed to produce eggs contained excessive amounts of a component possessing the electrophoretic mobility of the female protein. Comparisons of the female and carotenoid proteins thus established that the moth oocyte does not accumulate all proteins in proportion to their concentrations in the blood, but that the transport mechanism is characterized by a high degree of selectivity.

Selectivity of accumulation suggests that the protein transport mechanism may be more complex than could be accounted for alone by free diffusion from the blood to the oocyte. In two other cases of protein transport, it has been necessary to conclude that a protein combines temporarily with some element of the transport mechanism. Such a step has been postulated in order to explain the selectivity and competition demonstrated in the transmission of maternal antibodies to the foetus and the new-born of a number of species of mammals (Brambell, Halliday and Morris, 1958). In addition, adsorption of protein on the surface of an amoeba undergoing pinocytosis is convincingly suggested by kinetic and cytological evidence (Schumaker, 1958; Brandt, 1958).

Several mechanisms could be proposed to account for the selectivity of protein accumulation by the oocyte of the moth. Included among these is a temporary adsorption on the cell surface, as has been proposed for the amoeba. Adsorption on an intracellular structure such as the yolk spheres is not ruled out, however, nor is selective permeability of one of the various cellular envelopes which separate the oocyte from the blood. Distinguishing which of these possibilities may be correct must await further characterization of the uptake mechanism.

A final point which the protein accumulating mechanism of the moth oocyte may have in common with that of the amoeba is the intracellular disposition of the acquired protein. In both cases, the protein is associated to a major extent with cytoplasmic particles: pinocytic vacuoles in the amoeba and yolk spheres in the oocyte. Proteins with the antigenic properties of serum proteins are also associated with the yolk platelets of the amphibian oocyte (Cooper, 1948; Flickinger and Rounds, 1956). It may thus prove possible to establish a homology between these cytoplasmic structures with regard to their function in protein accumulation from the environment.

SUMMARY

1. At least seven proteins detectable by immunological techniques in the blood of the *Cecropia* moth have antigenic counter-parts in the oocytes produced by the female moth. While several oocyte antigens are undetectable in the blood of the female pupa, all of the proteins thus far observed in the blood are also present in the oocyte. Four out of five proteins of non-insectan origins, after being injected into the blood of the *Polyphemus* moth were detectable in the oocyte. Thus, the oocyte appears to accumulate almost all of the proteins present in the blood.

2. When the blood of *Cecropia* is injected into females of the *Polyphemus* moth, antigen 7, the "female protein," and antigen 3, a carotenoid protein, become distributed between the blood and the oocytes of the host in a manner quantitatively similar to their normal distribution in *Cecropia*. These two oocyte proteins may therefore be derived exclusively from the blood, rather than being synthesized to an appreciable extent in the ovary. The capacity of these proteins to combine with homologous antibodies was not detectably altered during their transmission from the blood to the oocyte.

3. Measurements of the concentrations of several proteins in the oocyte, relative to their concentrations in the blood, indicate that the mechanism of protein accumulation is selective. Of the proteins studied, the female protein was the most avidly accumulated, while the non-insectan proteins were detectable in the oocyte in relatively small amounts.

4. The proteins derived from the blood are probably localized primarily in the yolk spheres of the oocyte. Protein accumulation in this case therefore entails both the penetration of the oocyte surface and association with a cytoplasmic particle.

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THE EFFECTS OF BLOOD PROTEIN DEPLETION ON THE GROWTH OF THE OOCYTES IN THE CECROPIA MOTH

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During the final two weeks in the metamorphosis of the Cecropia moth the oocytes enter a period of yolk formation, in the course of which their volume increases 400-fold. The growth of the oocyte during this period is not dependent exclusively on the synthetic activities of the ovary, but also entails the transfer of proteins from the blood to the yolk (Telfer, 1960).⁻ An analysis of the mechanism of protein transfer and its role in the formation of yolk has been undertaken. We describe here some effects of depleting the blood of two prospective yolk proteins on the growth of the oocyte and on the composition of its yolk. Advantage was taken of the decrease which normally occurs in the concentration of the two proteins during the period of yolk formation.

The growth rate of the oocyte is interpreted here as an index to its rate of yolk formation. The Cecropia oocyte belongs to that class of eggs in which the yolk inclusions constitute the primary mass of the cytoplasm: centrifugation at $20,000 \times g$ yields a stratum of protein yolk spheres which occupies more than 90% of the volume of the oocyte. In such a case, gross changes in the rate of yolk formation should be conspicuously reflected in the growth rate of the oocyte.

Determinations of the growth rate of an oocyte, as with other internal structures of an organism, are handicapped by difficulties in measurements of the time axis. In the present case, it would be desirable to measure the individual oocyte at successive times in its period of yolk formation. However, the oocyte must be dissected from the moth in order to be measured and its capacity for further development is thereby destroyed. A second approach would be to measure the size of the oocytes in a large number of ovaries which varied only in the chronological stage of their development, and this procedure proved feasible for the Cecropia oocyte. The correlation between ovarian development and the stage of the moth's metamorphosis was found to be sufficiently precise so that we were encouraged to estimate the time of yolk formation from the directly observable time of metamorphosis.

METHODS

1. *Determination of the time of development*

The sequence of developmental changes used in determining the time of the moth's metamorphosis is described in Table I. The earliest developmental stage recognized with precision was the darkening of the tarsal claws which can be seen through the pupal cuticle with the aid of a dissecting microscope. In Schneiderman and Williams' (1954) timetable for the development of Cecropia males, tarsal claw

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darkening is listed as occurring 13 days after the initiation of the pupa's development into the moth. We arbitrarily designated this stage as day 13 for the female also, and subsequent stages of development were timed accordingly. Our timetable is based on the rate of development at 25° C., the temperature at which all animals were maintained.

2. *Dissection of the ovaries and determination of oocyte volume*

The ovaries were dissected from 70 females in the last half of their transformation from pupae into moths. The weights of the animals dissected were within the range of 4.5 to 5.5 grams, and it should be emphasized that the timing and magnitude of the changes described are probably valid only for animals within this size range. The final stages of the dissection and measurements of the size of the oocytes were completed with the ovaries immersed in 0.15 *M* NaCl. While the measurements were generally completed within an hour after the dissection had

TABLE I

Characters used in determining the time of development of the female pupa into a moth

Days of development at 25° C.	Diagnostic characters
13	Tarsal claws darken
13-14	Antennal rami darken
15-16	Spines on genitalia turn from white to reddish brown
16-17	Developing ovarian tracheae become lined by a cuticular thread demonstrable by teasing the tracheae apart
18	Ovarian tracheae become air-filled and thus appear silvery
18-19	Initiation of fore-wing pigmentation: lateral spots appear
19	Advanced wing pigmentation; lateral abdominal pigmentation visible
21	Cuticle softened over facial region
22	Cuticle crisp
23	Eclosion

been begun, neither swelling nor shrinking of the oocytes was visible in ovaries which had remained for as long as 4 hours in the saline solution.

The paired ovaries contain a total of 8 tubular ovarioles, one of which was selected for study from each animal. Since there was little obvious variation among the ovarioles with regard to the number or size of the oocytes they contained, the ovariole to be studied was selected at random. The only exceptions to this procedure were the omission of occasional ovarioles which obviously differed from their seven partners.

A single *Cecropia* ovariole generally contains from 30 to 50 oocytes in varying stages of development (Fig. 1). Their volumes were estimated, beginning with the most mature oocyte, which is located at the posterior end of the ovariole, and ending with the smallest oocyte which contained visible yolk. The presence of yolk was recognized through a dissecting microscope by the fact that it renders the cytoplasm opaque and colors it yellow. The termination of growth coincides with the secretion of the chorion. This event was detected by two criteria: since the chorion is opaque and colorless, it causes the oocyte to appear progressively paler as it is laid down over the yellow yolk; in addition, the chorion forms a rigid crust on the surface of the oocyte which thus loses its elastic response to probing.

The volume of an oocyte was calculated from measurements of its linear dimensions. The shape of a growing oocyte approximates an ellipsoid with two equal axes. The disparate axis is the longitudinal one, or that which coincides with the axis of the tubular ovariole (Figs. 1 and 2). Although the nurse cells accompanying each oocyte tend to flatten its anterior end (Fig. 2) and thus cause some deviation from the shape of an ellipsoid, this effect is progressively reduced as the oocyte enlarges and is undetectable during the last three-quarters of the volume increase. Therefore, the volume of a growing oocyte could be estimated by measuring its longitudinal and transverse diameters with a filar micrometer and by solving the appropriate equation for the volume of an ellipsoid.

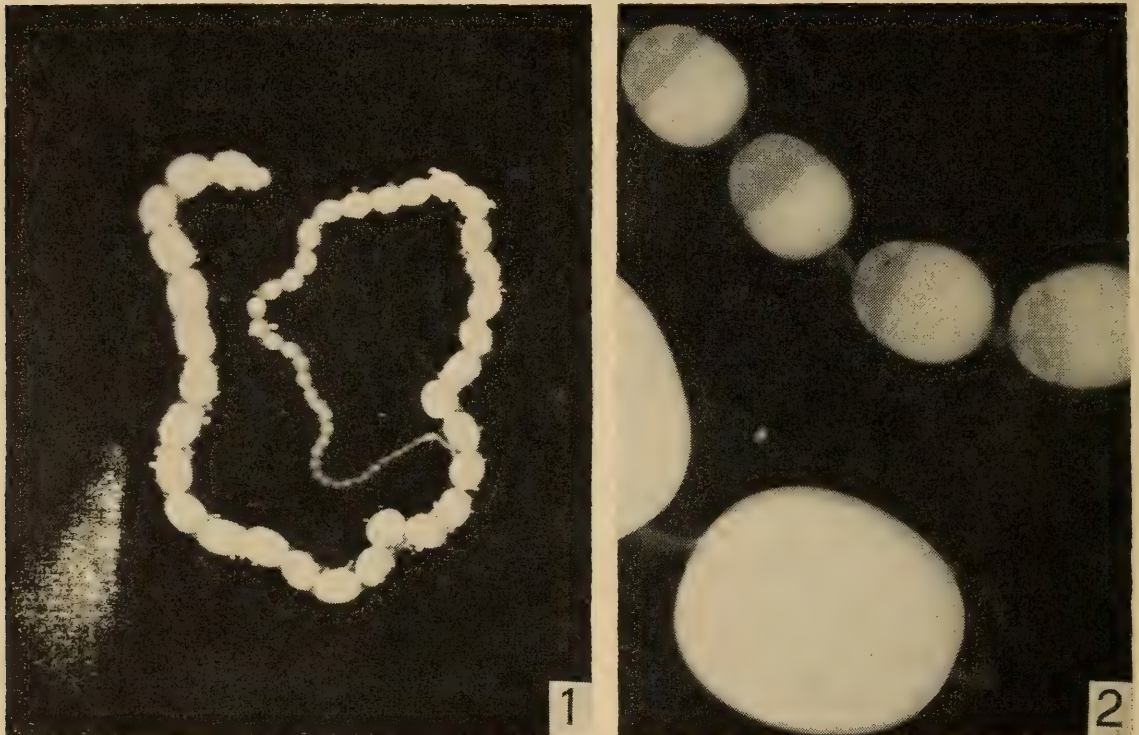


FIGURE 1. An ovariole dissected from a female *Cecropia* on the seventeenth day of its transformation from a pupa into a moth. The individual oocytes lie in sequence with the largest at the posterior end of the ovariole. Small fragments of fat body adhere to the outer wall of the ovariole.

FIGURE 2. Several oocytes which were removed from the ovariole shown in Figure 1. The five nurse cells form a transparent cap at the anterior end of the smaller oocytes. At the lower left is an oocyte which is nearing maturity. Magnification, $20\times$.

The most convenient index to the size of the mature oocyte was its weight, as determined with a semi-micro balance. Weighing was possible in this case because the rigid and comparatively impervious chorion allows the egg to be blotted and weighed without the loss of significant quantities of water from the oocyte itself.

As in the case of the growing oocyte, the volume of a mature oocyte could also be calculated from measurements of its diameters. The shape of the mature oocyte differs from that of the growing oocyte in that its two transverse diameters

are unequal. By means which are not apparent, one of the transverse diameters increases at the expense of the other during or shortly before the process of chorion formation. The longitudinal diameter appears to be unaffected by this process. Thus, the volume of a mature oocyte was calculated with the formula for the volume of an ellipsoid with three differing diameters.

3. *Determinations of blood protein concentrations*

The concentrations of two antigenically defined proteins in the blood and in oocyte extracts were determined with the antiserum-agar technique of Oudin. The two proteins have been described previously (Telfer and Williams, 1953). They included antigen 3, one of several carotenoid proteins in the blood, and antigen 7, a sex-limited blood protein which occurs primarily in female pupae and moths. The validity of the Oudin technique for the determination of the relative concentrations of these proteins has been discussed elsewhere, and in the case of the female protein, has been confirmed by independent methods (Telfer, 1954).

4. *Ovarian transplantations to males*

Comparisons of the effects of male and female blood on the size of the oocytes produced were made by transplanting ovaries to males which were then transfused with samples of either male or female blood. These experiments were performed on pupae of the Polyphemus moth. The method of CO₂ anaesthesia and other surgical procedures used are described in detail by Williams (1959). The tip of the abdomen was cut off from each male pupa and up to 0.5 of blood was removed through the resulting orifice by compressing the anterior end of the pupa. An ovary with a small amount of associated fat-body was dissected from a female pupa and inserted through the abdominal orifice in the male. The haemocoel of the male was then filled from a pipette with blood which had been obtained either from female pupae or from other male pupae. Finally, a piece of plastic cover-slip was sealed over the opening with paraffin. The implanted ovary was dissected from the host at the conclusion of metamorphosis.

RESULTS

1. *The volumes of the oocytes in individual ovarioles*

The stage of development of an ovariole and its oocytes can be described by plotting the volume of the successive oocytes as a function of their position in the ovariole. Three examples of the relationship in the developing *Cecropia* ovariole are shown in Figure 3. In all three cases oocyte volume decreased progressively along the sequence of oocytes. Exceptional in this respect were the volumes of the first 12 oocytes in the moth which had been developing for 20 days. The fact that these oocytes were nearly equal in volume can be attributed to their having completed growth, as was indicated by their all having undergone some degree of chorion formation. The three examples typify the gradient in developmental stage seen in an ovariole during the period of yolk formation. Profiles such as these for animals in varying stages of metamorphosis contain the information required for constructing growth curves for the oocyte in any particular numerical position.

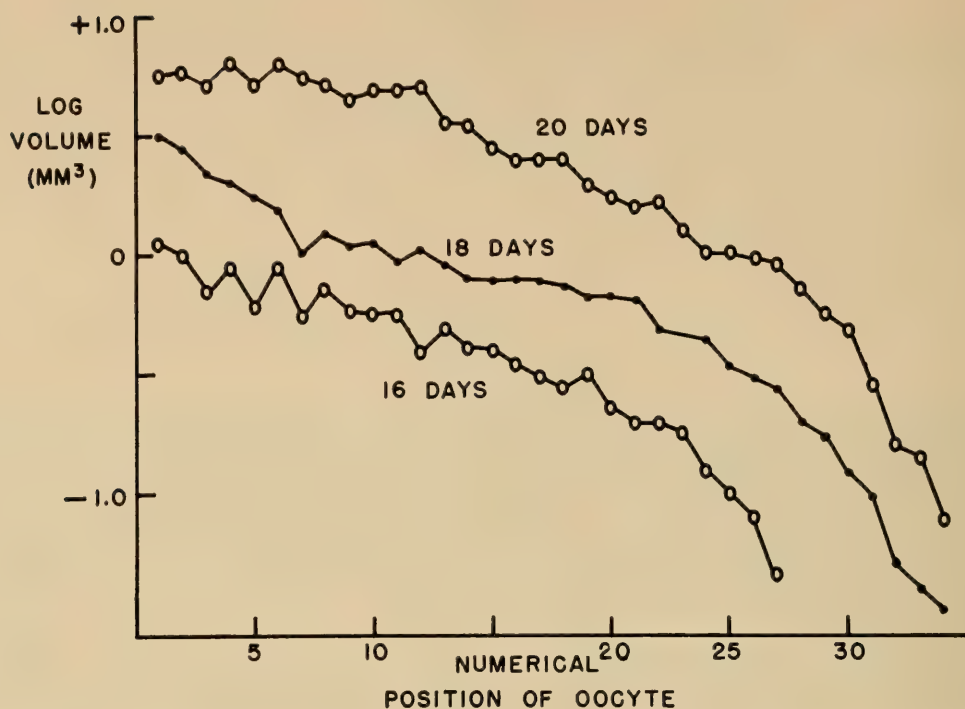


FIGURE 3. The logarithm of the volume of successive oocytes in three ovarioles. The stage of development of the female from which the ovariole was dissected, is indicated next to each curve and is expressed in days at 25° C. since the initiation of the pupal-adult molt.

2. Oocyte growth curves

The volume of the first, or most mature oocyte in each ovariole studied was plotted as a function of the morphologically determined age of the insect from which it had been dissected. The resulting data indicated that the logarithm of oocyte volume increases approximately linearly with time (Fig. 4) and then ceases abruptly at the time of chorion formation. Thus the growth of the first oocyte is described here as follows:

$$V = ae^{2.3bt}, \text{ or } \text{Log } V = \text{Log } a + bt, \quad (1)$$

in which V is the volume of the oocyte in mm^3 , t is the stage of metamorphosis expressed in days at 25° C., and a and b are constants. Analysis of the data by the methods described in Table II yielded the information that, during the one-week period for which data were obtained, the percentage relative growth rate of the first oocyte was $65 \pm 6\%$ per day while its percentage increment in volume was 91% per day. Thus, the first oocyte nearly doubles its volume on each of at least 7 successive days of yolk formation.

3. Comparisons of successive oocytes in the ovariole

Growth curves constructed for the tenth, twentieth and thirtieth oocytes were similar in form to that of the first oocyte. However, the growth constants differed in such a manner as to suggest that yolk formation is less vigorous in the more anteriorly located oocytes.

Each oocyte completes its growth at a later time than its more posterior neighbor. From the data on the time at which yolk formation is completed

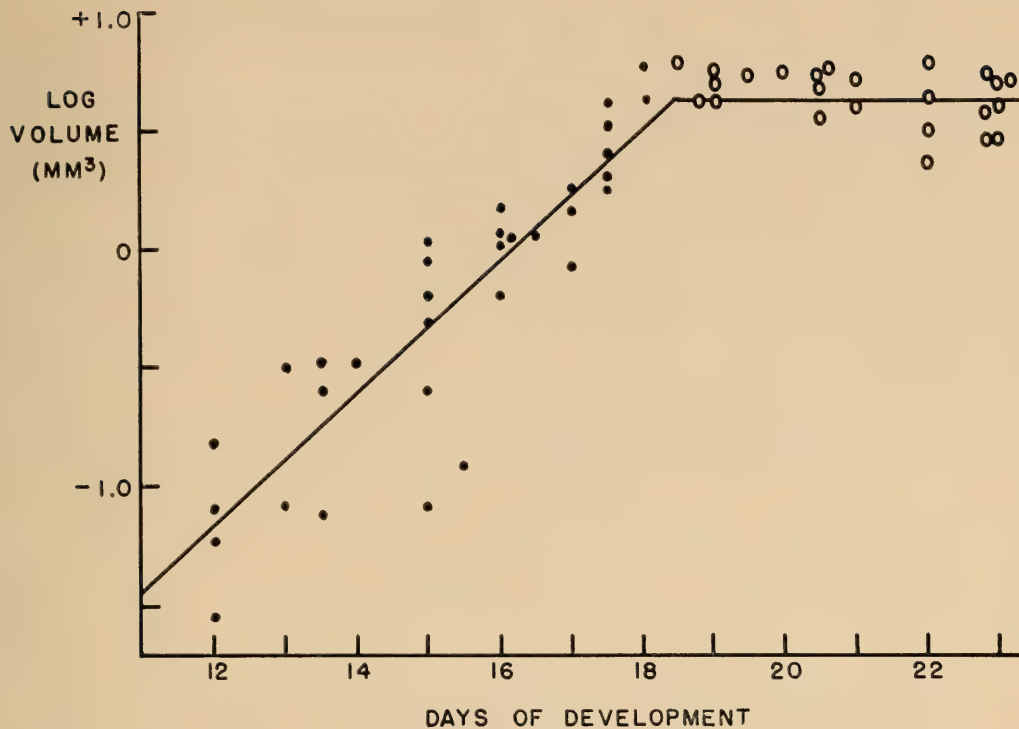


FIGURE 4. Growth curve of the first or most posterior oocyte in the ovariole of *Cecropia*. The logarithm of the volume of the oocyte is plotted against the time of development of the moth. Chorionated oocytes, —○—; oocytes without chorions, —●—.

(Table II), it can be calculated that in the ovariole as a whole, each oocyte completes yolk formation an average of 5.2 hours after its predecessor. However, there is a tendency for the first oocytes produced to follow each other in more rapid succession than the later oocytes. The average lag between successive oocytes was 4.2 hours for the first 20 oocytes, and was estimated to be 7.2 hours for the twentieth to thirtieth oocytes.

Also varying with numerical order in the ovariole is the volume of the oocyte at maturity. The volume of the first oocyte was 130% of the volume of the tenth

TABLE II
Growth characteristics of oocytes during yolk formation

Growth characteristic and its method of calculation	Numerical position in ovariole			
	1	10	20	30
Mean volume ($\pm$ s.e.) of oocytes possessing chorions (mm. ³)	4.75 $\pm$ 0.25	3.64 $\pm$ 0.13	3.35 $\pm$ 0.46	3.0
<i>b</i> (equation 1); calculated by the method of least squares for the non-chorionated oocytes	0.28	0.23	0.21	0.18
Percentage daily increment in volume [(Antilog <i>b</i>) - 1] 100%	91%	70%	62%	51%
Percentage relative growth rate (2.3 <i>b</i>) 100% (% per day)	65%	53%	48%	41%
Time when growth terminates: the value of <i>t</i> (equation 1) when <i>V</i> is the mean volume of the oocytes possessing chorions (days)	18.6	20.1	21.9	24.9

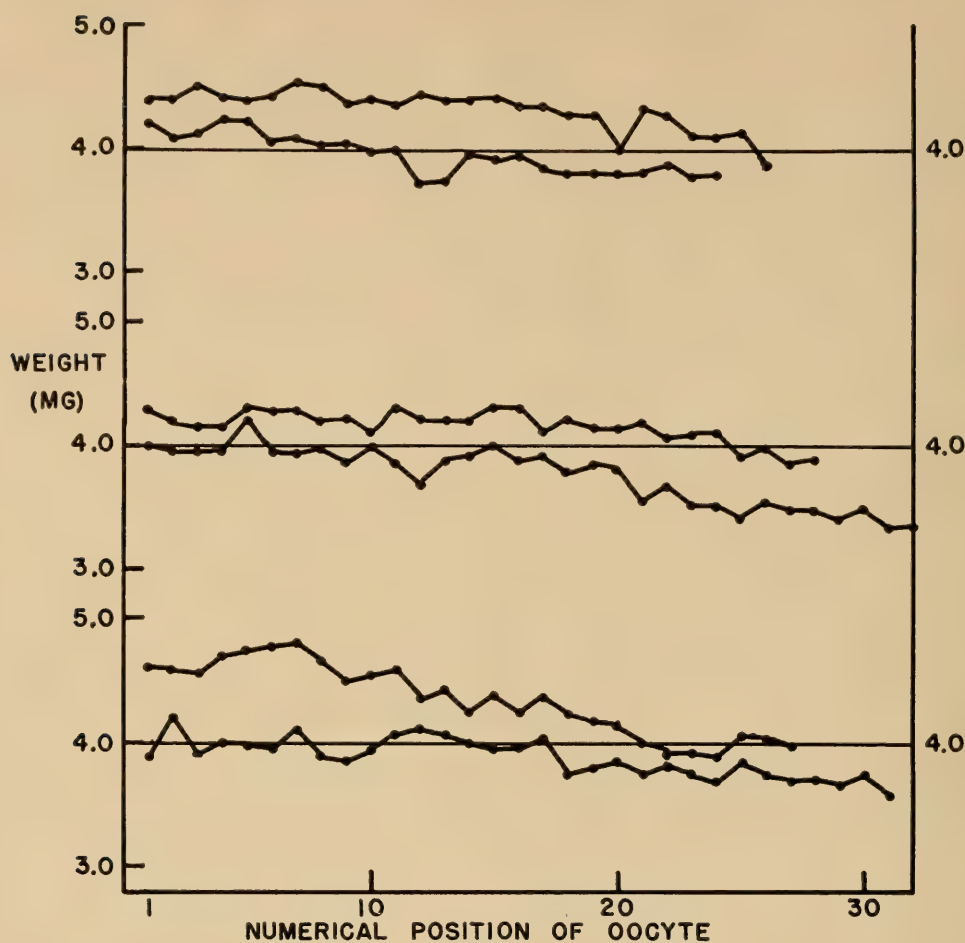


FIGURE 5. The weights of mature oocytes dissected from moths which had completed metamorphosis. The weights of successive oocytes from six different moths are plotted as a function of their numerical position in the ovariole. The reference lines are arbitrarily placed at 4.0 mg.

oocyte and 142% of that of the twentieth oocyte. The number of chorionated thirtieth oocytes was too small to permit the comparison to be extended to this case. The relationship between size at maturity and position in the ovariole was seen in a more exact manner by weighing the successive oocytes in mature ovarioles dissected from fully developed moths. In all six cases illustrated (Fig. 5), the later oocytes tended to be smaller than the first oocytes produced.

TABLE III

Comparison of the b-value for the first oocyte with those for numbers 10, 20 and 30

Numerical position of oocyte	$b \pm \text{s.e.}$	N	Significance of difference from first oocyte (p)
1	0.28 ± 0.025	33	—
10	0.23 ± 0.025	41	0.11
20	0.21 ± 0.023	43	0.04
30	0.18 ± 0.026	28	0.008

Chi square test of the significance of the over-all decline; $p = 0.003$.

The value of b , and consequently the rate of growth, also decreases with increasing numerical position (Table II). The difference in b between the first and the thirtieth oocytes represents a decline in the percentage relative growth rate from 65% to 41% per day. Statistical comparisons indicated that the decline observed in b was significant (Table III).

Thus, all of the growth characteristics considered here change in such a manner as to suggest that yolk formation occurs with reduced vigor in the later oocytes produced in an ovariole. The question then arises as to whether this is a direct consequence of a depletion in the supply of prospective yolk proteins available in the blood.

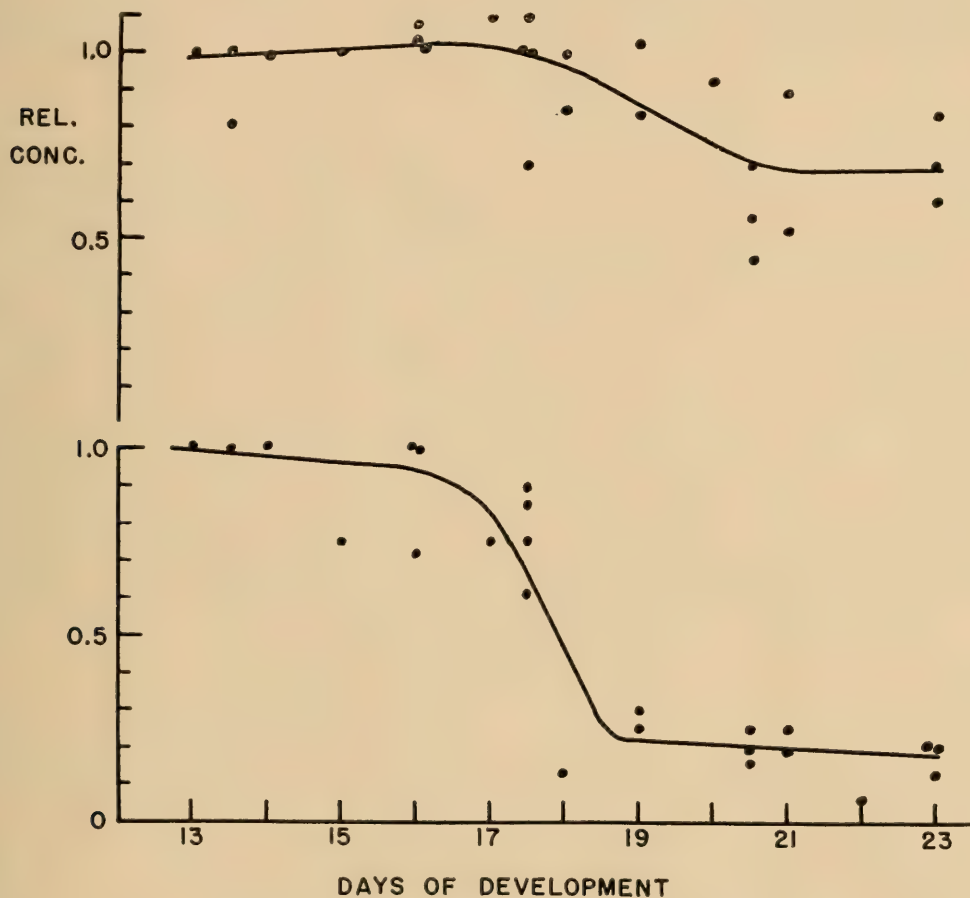


FIGURE 6. Changes in the relative concentrations of two proteins in the blood during the last ten days of the pupal-adult transformation. Upper curve is for antigen 3, a carotenoid protein. Lower curve is for antigen 7, the female protein. A relative concentration of 1.0 is arbitrarily set as the maximum concentration of each protein in the blood.

4. *Changes in the concentrations of two blood proteins during yolk formation*

Most, and possibly all, of the moth's blood proteins are deposited in the yolk to some extent, and of those studied quantitatively, the female protein is the most avidly accumulated (Telfer, 1960). It would seem reasonable, therefore, that depletion of the available blood proteins, and of the female protein in particular, could affect the growth characteristics of the oocytes. That the concentration of the female protein in the blood decreases during the last half of the transformation

of the pupa into a moth has already been demonstrated (Telfer, 1954). A more precise determination of the timing of the decrease is described here, as well as a comparison with the less readily accumulated carotenoid protein.

The results indicate (Fig. 6) that the concentration of female protein decreases approximately 80% during the period of yolk formation. The concentration of the carotenoid protein in the blood also drops during yolk formation, but in this case the decrease is not nearly as great. The difference between the two proteins in this regard is consistent with the greater extent to which the oocyte accumulates the female protein.

The timing of the decrease in female protein with relation to the growth of the four oocytes studied is summarized in Figure 7. While the first oocyte undergoes the final three-quarters of its growth (left hand cross-hatched area in Figure 7)

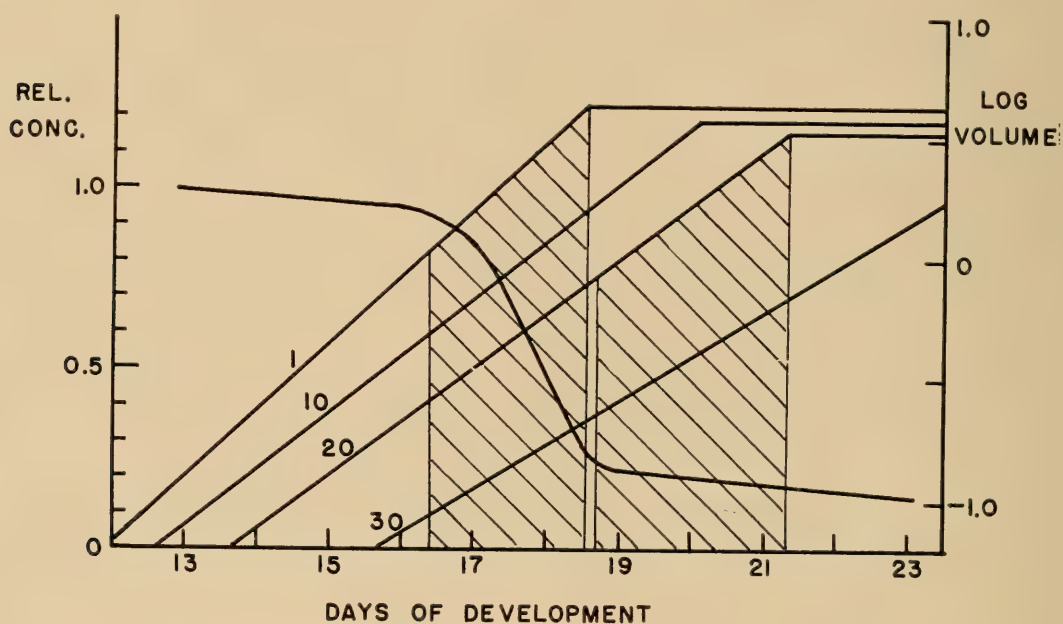


FIGURE 7. Changes in concentration of the female protein (left-hand scale) superimposed on the growth curves of the first, tenth, twentieth and thirtieth oocytes (right-hand scale). Cross-hatched areas indicate the time during which the first and twentieth oocytes undergo the final three-fourths of their volume increase.

the average female protein concentration approximates one half or more of its maximum level in the blood. The twentieth oocyte, on the other hand, undergoes the final three-quarters of its volume increase (right hand cross-hatched area) when the blood contains only one-fifth of its maximum concentration of female protein. It thus appears that the first oocytes are produced in an environment in which the concentration of female protein is greater than that of the later oocytes, and we are left with the possibility that this accounts for the reduced vigor with which the later oocytes produce yolk.

5. *The effects of a protein's concentration in the blood on its rate of accumulation by the oocyte*

The most likely mechanism by which a reduction in blood protein concentration could retard the process of yolk formation would be through a decrease in the rate of protein accumulation by the oocyte. We therefore sought to determine whether

the rates of female and carotenoid protein accumulation are affected by the decrease which normally occurs in the concentrations of these proteins in the blood. The information required here, in addition to the growth rates of the successive oocytes, was the amount of each protein accumulated per mg. of oocyte.

Successive pairs of chorionated oocytes dissected from fully matured ovarioles were weighed and then extracted with 0.2 ml. of 0.15 *M* NaCl buffered at neutrality. Either the oocytes or the extracts were frozen and thawed once, a

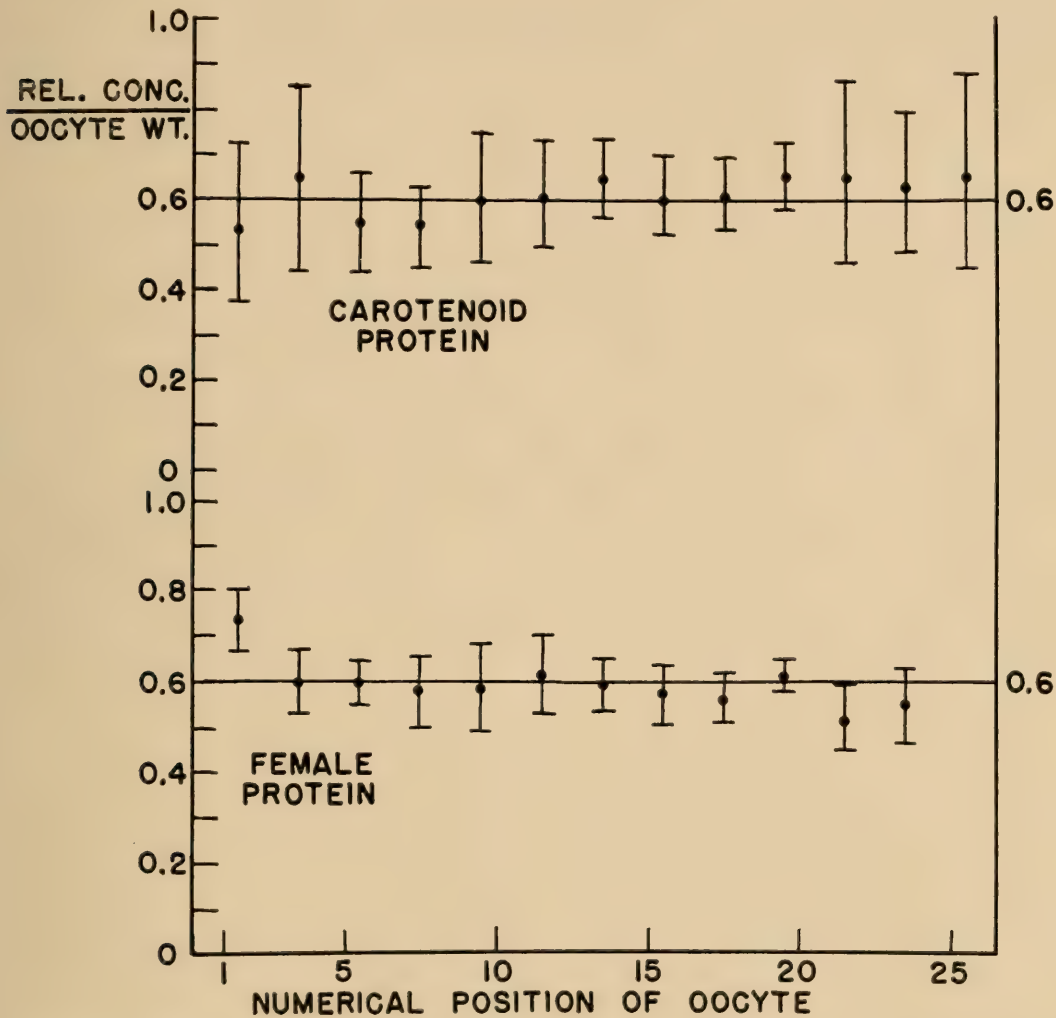


FIGURE 8. Relative concentrations of the carotenoid and female proteins in successive pairs of oocytes dissected from mature moths. The points represent the mean values for five different animals. The bars indicate 95% confidence intervals. The relative concentration of the protein in an extract of standard volume was divided by the fresh weight in milligrams of the oocytes before extraction. Reference lines are arbitrarily placed at 0.6.

procedure which has been found to lyse the yolk spheres and thus to release their stored proteins. The concentrations of female and carotenoid proteins in the extracts were determined with antiserum-agar tests.

From the results summarized in Figure 8, it is apparent that the amount of female protein extracted per mg. of mature oocyte tended to remain constant along the length of the ovariole. While there may have been some decline, this was of questionable significance and it did not approximate the 80% decrease in the

concentration of female protein which occurs in the blood. A similar relationship was found for the carotenoid protein. Despite the approximate 20% decrease in the level of carotenoid protein in the blood, the concentration of this protein in the oocyte tended to remain constant, and perhaps even rose slightly, along the length of the ovariole (Fig. 8).

When considered independently, these results suggest that the rates of accumulation of the two proteins are not affected by the decrease in their concentrations in the blood. However, a comparison of growth rates indicates that the time available for protein accumulation is longer in the later oocytes than in the first oocyte. For instance, the twentieth oocyte required 12 days to increase in volume from 0.01 mm.³, the volume of the smallest oocytes observed to contain yolk, to 3.35 mm.³, its volume at maturity. Due to its higher growth rate the first oocyte requires only 9 days for the same increase in volume. Thus, while the concentration attained by the proteins may be the same in the two oocytes, the time required to achieve this concentration is 33% longer in the twentieth oocyte.

It therefore seems likely that naturally occurring reductions in the concentrations of the carotenoid and female proteins in the blood result in their being accumulated by the oocyte at slower rates. This, in turn, could account for the slower growth rate and smaller size of the oocytes which produce yolk at the end of metamorphosis. However, alternative explanations are possible; for instance, the results summarized in Figure 8 would also be expected if the rate of protein accumulation were governed primarily by the rate at which space is provided for the protein in the oocyte. In such a case, the rate of protein accumulation would be determined by the growth rate of the oocyte, which is the inverse of the relationship proposed above. It therefore proved desirable to seek an independent test of the extent to which deficiencies of a blood protein can impair the growth of the oocyte.

6. *Oocytes produced by ovaries transplanted to males*

Experimental evidence that the level of female protein in the blood may affect the growth of the oocyte was derived from ovarian transplantation experiments. Kopeć (1911) observed in a number of Lepidoptera that the oocytes produced by ovaries which had been transplanted to males are considerably smaller than those produced by ovaries in females. A similar effect has been observed in *Cecropia* (Telfer, 1954) and is demonstrated here for the *Polyphemus* moth. The following experiments indicate that this effect is due to the male's deficiency in a factor which is present in the blood of female pupae.

Single ovaries dissected from diapausing female pupae of the *Polyphemus* moth were transplanted to the haemocoel of diapausing male pupae. Half of the male pupae were then injected with the blood of male pupae and the other half with the blood of female pupae. The volume of the blood injected was such that the recipient's blood contained a $\frac{1}{5}$ to $\frac{1}{10}$ dilution of the injected blood. Fifteen of the males survived the surgical procedures and completed their development into moths. In 13 of these the implanted ovary, which was dissected from the moth, contained oocytes that had completed their development, as was indicated by the presence of a chorion.

The presence of blood from female pupae enhanced the size attained by the oocytes developing in males. In the males transfused with male blood the average

weight of the mature oocyte was 2.5 mg., the values ranging from 1.9 to 2.8 mg. In those transfused with female blood, the average weight was 3.6 mg. with a range of 2.2 to 4.8 mg. The variance in the latter case was significantly greater ($p = 0.04$) than that for the male-transfused animals—a fact which suggests that there were inconsistencies in the effective level of female blood received by the host. In any case, 6 of the 8 males transfused with female blood produced oocytes which weighed 3.8 mg. or greater and which were thus within the size range of oocytes produced by normal ovaries in *Polyphemus* females.

Blood taken from female pupae—a stage, incidentally, during which yolk formation does not occur—thus contains something which is lacking in the male and which enhances the formation of yolk. That the female protein is the responsible agent is suggested by the fact that it is approximately one thousand times more concentrated in the blood of female pupae than of males (Telfer, 1954), and that it is accumulated by the growing oocytes in substantial quantities.

DISCUSSION

1. *Comparison with yolk formation in other animals*

The growth rate of an oocyte might be expected to serve as a valid index to the rate of yolk formation only in oocytes whose cytoplasm becomes nearly close-packed with yolk bodies—as in the case of moths and birds. Even in moderately yolky oocytes, however, yolk formation, which is limited to the final stages in the total differentiation of the oocyte (Wilson, 1925), may be the primary cause of the volume increase occurring at the time.

Despite the hazards in this interpretation, comparisons of the *Cecropia* oocyte with several other oocytes whose growth has been described indicate clearly that the rate of yolk formation varies widely from species to species. Although, to our knowledge, the growth of the amphibian oocyte has not been the subject of an analysis in which the parameter of time was measured, one can presume that its growth during yolk formation is relatively slow from the fact that it spends several months in the process (*cf.*, Grant, 1953). By contrast the process is rapid in the chicken and in the fruitfly. In chickens the absolute growth rate of the oocyte during yolk formation reaches an astonishing 5 grams per day, while the maximum percentage increment in volume has been measured at 284% per day (Romanoff and Romanoff, 1949). The relatively minute oocyte of *Drosophila melanogaster* completes yolk formation in an estimated 4.7 hours and during this time its volume doubles approximately once every 0.7 hours (King, 1957). The *Cecropia* oocyte with its daily increment in volume of 50–100% per day appears to be intermediate between these extremes.

Variations such as these are presumably adaptations to various factors in the over-all economy of the organism. Whatever their explanation, it is apparent that yolk formation can be a rapid process, resulting in both absolute and relative growth rates which may be among the fastest for metazoan cells.

2. *Conclusions concerning the mechanism of yolk formation in insects*

Data concerning the growth of the oocyte in *Drosophila melanogaster* (King, 1957) are sufficiently extensive to allow a detailed comparison with that of *Cecropia*. The growth curves of these two oocytes during their period of yolk formation have

two characteristics in common, one of which is the abruptness with which growth terminates. There is no period of senility detectable in the yolk-producing mechanism of insects. The correlation between the termination of growth and the first appearance of the chorion in *Cecropia* suggests that the two events may be causally related to each other.

A second similarity is the apparent exponential character of oocyte growth during yolk formation. A constant relative growth rate, which is implied by this result, is frequently found in biological systems and can in many cases be interpreted as indicating that the products of growth in turn reproduce. Such an interpretation is not plausible in the case of yolk formation; it would require that yolk be produced throughout the substance of the existing yolk mass, whereas evidence from a number of organisms with relatively yolky oocytes indicates that new materials are deposited primarily at the periphery of the cell.

Lipophilic dyes injected into the chicken (literature reviewed by Romanoff and Romanoff, 1949) and radioactive amino acids injected into frogs (Kemp, 1955) both localized at the periphery of the oocyte rather than throughout its substance. Evidence that the yolk spheres of the insect oocyte grow primarily at the periphery of the cytoplasm is suggested by the cytochemical localization of a number of constituents of the yolk (Bonhag, 1955, 1956; Telfer, 1958). These observations make it likely that the exponential increase in the yolk mass of the insect oocyte results, not from self-reproduction of the mature yolk bodies but from an exponential increase in a peripherally-located mechanism for the formation of yolk. Whether this mechanism is, simultaneously, a self-reproducing mechanism remains, however, a matter of conjecture.

If equation (1) is in fact an accurate description of the growth of the insect oocyte, an additional characteristic pertinent to investigations of the mechanism of yolk formation can be proposed. The volume of the *Cecropia* oocyte is approximately 400 times greater at the conclusion of yolk formation than at its inception. It follows that the absolute rate of yolk formation also increases by a factor of 400. If one assumes that the oocyte has an approximately constant shape during yolk formation, its surface area increases by a factor of $400^{2/3}$, or only 50. Thus a unit of surface area, or perhaps of the cytoplasm underlying the surface, must produce yolk 8 times faster just before the conclusion of yolk formation than just after its inception. Either the density or the rate of activity of the yolk-producing elements at the surface of the oocyte must also increase by a factor of 8, a fact which could prove useful in the cytological identification of the yolk-producing mechanism. Finally, a similar increase must occur in the rate of penetration per unit of oocyte surface area by blood proteins and by all other raw materials required in the formation of yolk.

3. *The role of blood protein accumulation in yolk formation*

As will be demonstrated more fully in a later paper, the blood proteins accumulated by the *Cecropia* oocyte are confined to the protein yolk spheres which comprise the bulk of the mass of the oocyte. The process of yolk formation in this insect thus entails the transfer of proteins across the cell surface and their incorporation into a cytoplasmic particle. It is understandable, therefore, that the growth of the yolk mass might be sensitive to the availability of blood protein.

Of two normally occurring blood proteins and five artificially introduced proteins thus far studied (Telfer, 1960), the female protein is the most avidly accumulated, attaining a concentration ratio (oocyte:blood) of 20:1. The evidence presented here suggests that deficiencies in this protein can impair the growth of the oocyte. Oocytes reaching maturity in the low female protein environment which prevails at the termination of metamorphosis grow at a slower rate and are smaller at maturity than oocytes which grow during an earlier and more bountiful period. In addition, oocytes developing in males which normally contain only traces of female protein in their blood attain a 40–50% larger size if the host has been injected with the blood of female pupae. There is thus a correlation between the availability of female protein in the blood and the size attained by the oocyte. Whether the correlation results from the influence of the female protein alone on yolk formation is a question whose answer must await the isolation of this protein in experimentally useful amounts. It is probable however that we are dealing here with the effects of a blood protein on the growth of the oocyte and the formation of yolk.

SUMMARY

1. The earliest oocyte to differentiate in an ovariole of the *Cecropia* moth grows during the final week of its development with a daily increment in volume which was calculated to be 91% per day. Growth during this period is due primarily to the accretion of yolk spheres. It terminates abruptly when the chorion appears at the surface of the oocyte.

2. Successive oocytes in an ovariole lag behind each other in their development by an average of approximately 5 hours; the first oocyte terminates growth more than 6 days earlier than the thirtieth oocyte. As a consequence, the first oocyte produces yolk at a time when at least two prospective yolk proteins are present in the blood at higher levels than those prevailing during the growth of the later oocyte.

3. Correlated with a decline in the level of the two blood proteins are reductions in the growth rates of successive oocytes in the ovariole and in the final size which they attain. Experimental results suggest that depletion of the female protein in the blood may be one of the primary causes of the reduced vigor with which the later oocytes grow.

4. The final concentrations of the two proteins were the same in successive oocytes in the ovariole. Thus, while the normally occurring depletion in the blood's supply of prospective yolk proteins may lead to a reduction in the rate and amount of yolk formation in an oocyte, it does not affect grossly the quality of the yolk produced.

5. From a consideration of the oocyte growth curve and of cytological observations reported for other yolk oocytes, it is proposed that yolk is formed in the *Cecropia* oocyte by an exponentially increasing mechanism which is probably located at the periphery of the cell.

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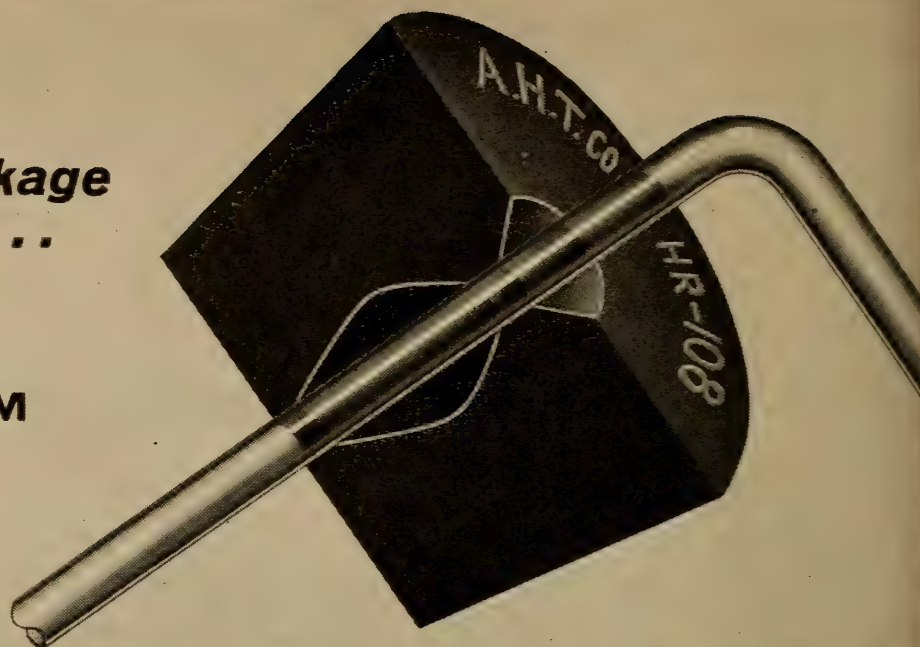
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MAGNETIC RESPONSE OF AN ORGANISM AND ITS SOLAR RELATIONSHIPS ¹

F. A. BROWN, JR., W. J. BRETT, M. F. BENNETT AND F. H. BARNWELL ²

Departments of Biological Sciences, Northwestern University, Indiana State Teachers College, and Sweet Briar College, and The Marine Biological Laboratory, Woods Hole, Mass.

It has become increasingly evident in recent years that the living organism is sensitive to fluctuations in still unidentified geophysical factors, in addition to those factors with which physiologists and ecologists have customarily concerned themselves in the past (Brown, 1959a). Such obvious environmental factors as temperature, light, humidity, gravity, pressure, and sound, are clearly ones toward which it is usually highly important that organisms exhibit immediate adaptive responses. Correspondingly, it is well-known that living things, in general, have their behavior and basic physiology dominated in terms of response to them.

On the other hand, organisms in environments held constant with respect to all these obvious factors still exhibit physiological and behavioral fluctuations correlated with fluctuations in other such geophysical parameters as atmospheric pressure, atmospheric temperature, primary cosmic radiation, and general background radiation, including both their regular atmospheric tidal changes, and their large, weather-related, essentially aperiodic, changes. The correlations are of such character as to indicate clearly that other and yet unidentified factors are also effective for organisms. These less obvious environmental factors appear able to induce or trigger relatively large biological alterations, not uncommonly into the range of 30% or more in their deviation from longer-term mean values. In general, it does not appear adaptive that organisms should permit physiological processes to be regulated by these subtle factors instead of by the more obvious environmental ones, and not unexpectedly, therefore, homeostatic mechanisms seem normally to be continuously operative in reaction to the alterations induced by them.

In two particular associations, however, such subtle environmental factors and their fluctuations may possess clear adaptive value, and there are good reasons to postulate that organisms have evolved physiological means for utilizing this value.

¹ This research was aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, #1228-03.

² The authors wish to acknowledge their indebtedness to Professor L. I. Bockstahler of the Department of Physics for his generous assistance with problems related to the magnetic field.

One of these is in temporal orientation, or the timing of the well-known temperature-independent solar-daily, lunar-daily, synodic monthly, and annual periodicities that commonly persist in complete constancy of all factors to which the organisms are usually deemed sensitive. Indeed, it has been shown possible to account for all the many and peculiar described properties of these long-cycle persistent rhythms in terms of periodicities effected by the subtle factors (Webb and Brown, 1959; Brown, 1959). The second association relates to spatial orientation, particularly as it pertains to the periodic migrations of organisms for feeding and breeding, and especially to the navigational aspects of the phenomenon.

Suggesting a role of subtle environmental factors in animal navigation is the demonstration in recent years of a very close interrelationship between the phenomena of temporal and spatial orientation of animals (Hoffman, 1954; Pardi and Grassi, 1955; Renner, 1959) quite as our own spatial orientation may depend on relationships to sun, moon or the constellations as a function of time. In both of these associations, temporal and spatial orientations, control by the obvious factors, when available, may be the dominating consideration. However, a number of observed characteristics of these phenomena appear inadequately accounted for in these terms alone. Such problems might be greatly simplified were it learned that organisms had available additional information.

On the one hand, it is obvious from available evidence that at least some subtle geophysical factors are perceived by organisms. And yet, on the other hand, cursory examinations for the biological effectiveness of changes in various ones of these within essentially their natural range have in the past yielded generally negative results. Therefore, it seems obvious that the question must be more critically re-examined. The work about to be described is the consequence of a brief, intensive re-examination for possible response to the magnetic field. In this study, reported earlier briefly (Brown, Brett and Webb, 1959; Brown, Bennett and Brett, 1959; Brown, Webb, Bennett and Barnwell, 1959), not only is it apparent that an organism responds significantly to the changes in the magnetic field through alterations in its spatial orientation, but that the response is intimately associated with the mechanism of temporal orientation of organisms quite as might be expected were the magnetic orientation to possess usefulness to the organism.

METHODS AND MATERIALS

The common mud-snail, *Nassarius obsoleta*, was used in this study. Collections were made at Chappaquoit Beach, West Falmouth, Massachusetts, at approximately weekly intervals, and the stock collections were maintained on a table in running sea-water in the ordinary daily illumination changes of the laboratory.

Simple equipment (Fig. 1) was constructed to determine the degree of right and left turning of snails as they emerged from a narrow corridor into a constant, symmetrically illuminated field. The apparatus consisted of an aluminum, funnel-shaped corral fastened in an 8-inch crystallizing dish set upon a polar-coordinate grid with the opening of the funnel over the center of the grid. A circle of 3 cm. radius was drawn. The grid was ruled into 22.5° sectors, and the long axis of the corridor was accurately aligned with the center of the sector labelled zero. Since the sizes of the snails varied from nearly the width of the corridor to half of that value, even snails following one or the other corridor wall and continuing on a straight path

would, after 3 cm., lie in the zero sector. Successive sectors to the right were given numbers + 1 through + 4, and to the left, - 1 through - 4. This apparatus was placed on the bottom of a $17 \times 12 \times 10$ inch box, as shown in Figure 2, in such a manner that a $4\frac{1}{2}$ -inch round window illuminated by an enclosed 60-watt incandescent lamp and covered with a white diffusing transmitter lay above and slightly ahead of the corridor exit. A hooded window for observing the snail movements, and for re-corralling the snails between the successive runs of ten, was

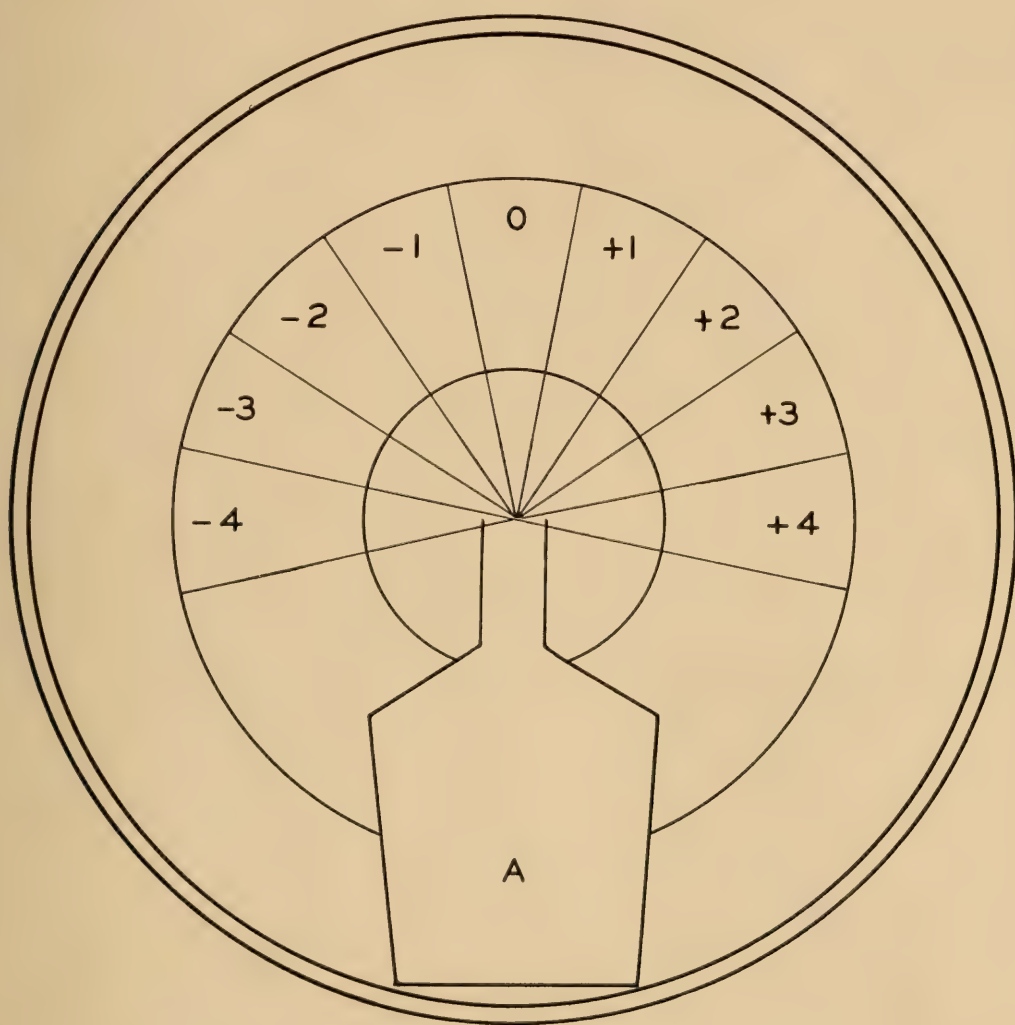


FIGURE 1. Orientation apparatus. View from above of the corral (A) in an 8-inch glass crystallizing dish placed over a polar grid. All dimensions may be scaled from the 3-cm. radius of the innermost circle.

located above and to the rear of the corral. Beneath the box was an adjustable platform with a horizontal turntable upon which an 18-cm. Alnico bar magnet could be easily placed, centered under the corridor exit and orientated in any compass direction, or from which the magnet could be removed quickly. The magnet at 14 cm. below the orientation chamber gave a horizontal intensity (1.5 gauss) only about nine times that of the earth's field in Massachusetts (.17 gauss). Such a weak experimental magnetic field was purposely selected to increase the probability that any response which might be discovered was a naturally occurring phenomenon.

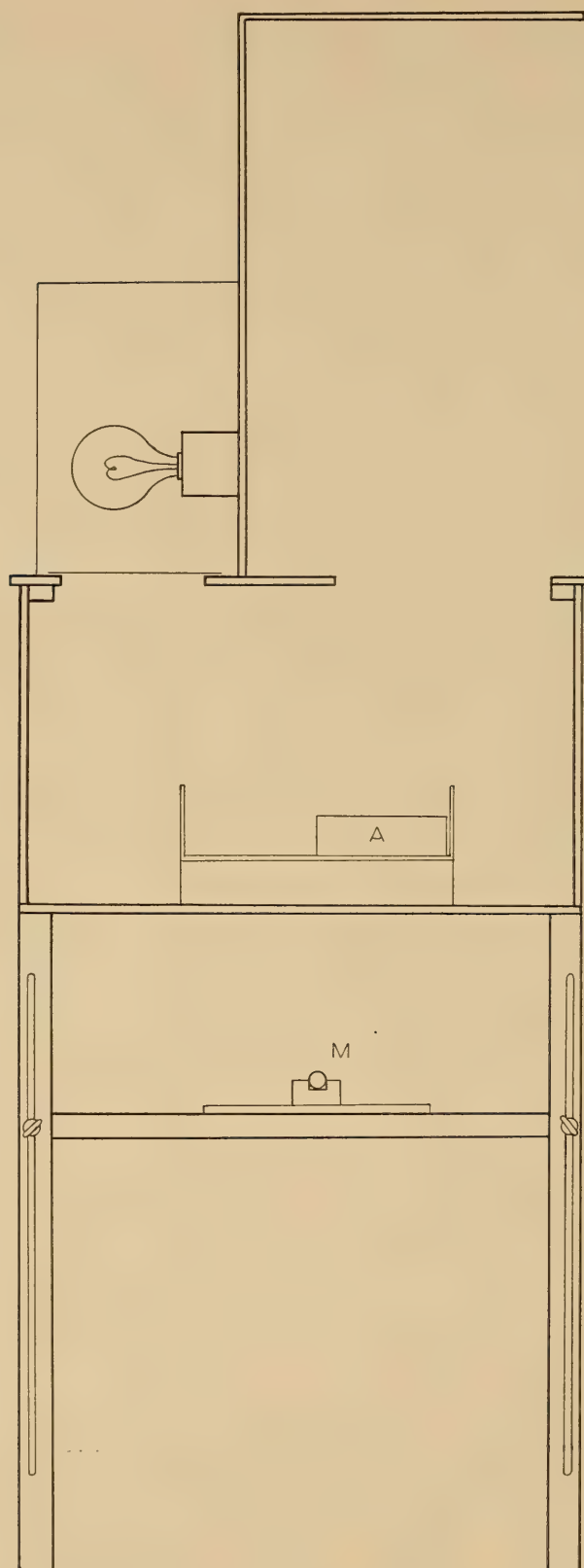


FIGURE 2. Lateral view of the diagrammatically-sectioned wooden orientation apparatus, drawn to scale. A is the corral, M is the bar-magnet.

Such a field strength would be expected to lie within the range of sensitivity of any normally operative responder system which might be present for magnetism. Four identical orientation chambers were available during the study.

The experimental procedures were kept uniform, simple and minimal. The apparatus was always oriented carefully so that the corridor exit was pointed toward the magnetic south. Sea-water to a depth of about 2 cm. was added to the crystalizing dish. Eleven to fifteen snails were dropped into the corral. Each completed experimental series consisted of 60 snail exits, two groups of ten exits without magnet, two groups of ten with the artificially increased magnetic field oriented as the earth's field (with north-seeking pole directed south), and two groups of ten with the north-seeking pole directed west. The first three groups of ten usually consisted of one of each of the three experimental conditions, as did also the last three. The order within the three groups of ten, however, changed continuously. Throughout most of the period of experimentation, the observer was uninformed as to the presence or absence, or orientation, of the magnet. Each snail emergence was accorded one number, that of the sector in which the head, or greater fraction of it, was located at the instant the snail reached the 3 cm. arc.

It is important to note that the method does not involve the determination of any final pathway chosen by the snails; rather, it simply yields a measure of any tendency toward change in direction from the initial path, and the relative degree of this tendency. The use of this kind of measurement appeared to reduce to a minimum obviously erratic, exploratory movements.

It was learned from brief exploratory experiments that the mean path of turning for any given time was not conspicuously influenced by changing the compass direction of orientation of the experimental chamber.

During the two-month period, June 28 to August 29, 1959, inclusive, 564 such series of 60 snail passages were obtained. Series were obtained for all hours of the day from 5 AM through 9 PM, Eastern Standard Time. No single one of these hours of the solar-day was represented by fewer than 9 or by more than 50 series of 60 snail exits.

RESULTS

It was apparent early in the experimentation that the snails, whether controls *without* experimentally imposed magnetic field or experimentals *with* such imposed fields, varied greatly from one time to another in both their spontaneity of activity and their rate of locomotion. This resulted in differences in time for completion of a single series of 60 runs, ranging from about 12 to 60 minutes. Secondly, the pattern of emergences of the snails clearly differed significantly from one time to another under the same experimental conditions. Thirdly, the results of comparisons within single series of 60 runs indicated an influence of the experimental magnetic fields.

Sample patterns of emergence of the snails are illustrated for nine series in Figure 3. These were selected, without reference to time, for the purpose of illustrating the wide variety of results. It is quite evident from this figure that the experimental magnetic fields, even if effective, could not be producing a simple uniform response. As seen from the sample patterns, the introduction of the N-S oriented magnet (B in figure) could, as the average of the two runs of ten snails, be associated with a mean path to left, (*e.g.*, #1, 2, 7, 9) or to the right (*e.g.*, #3,

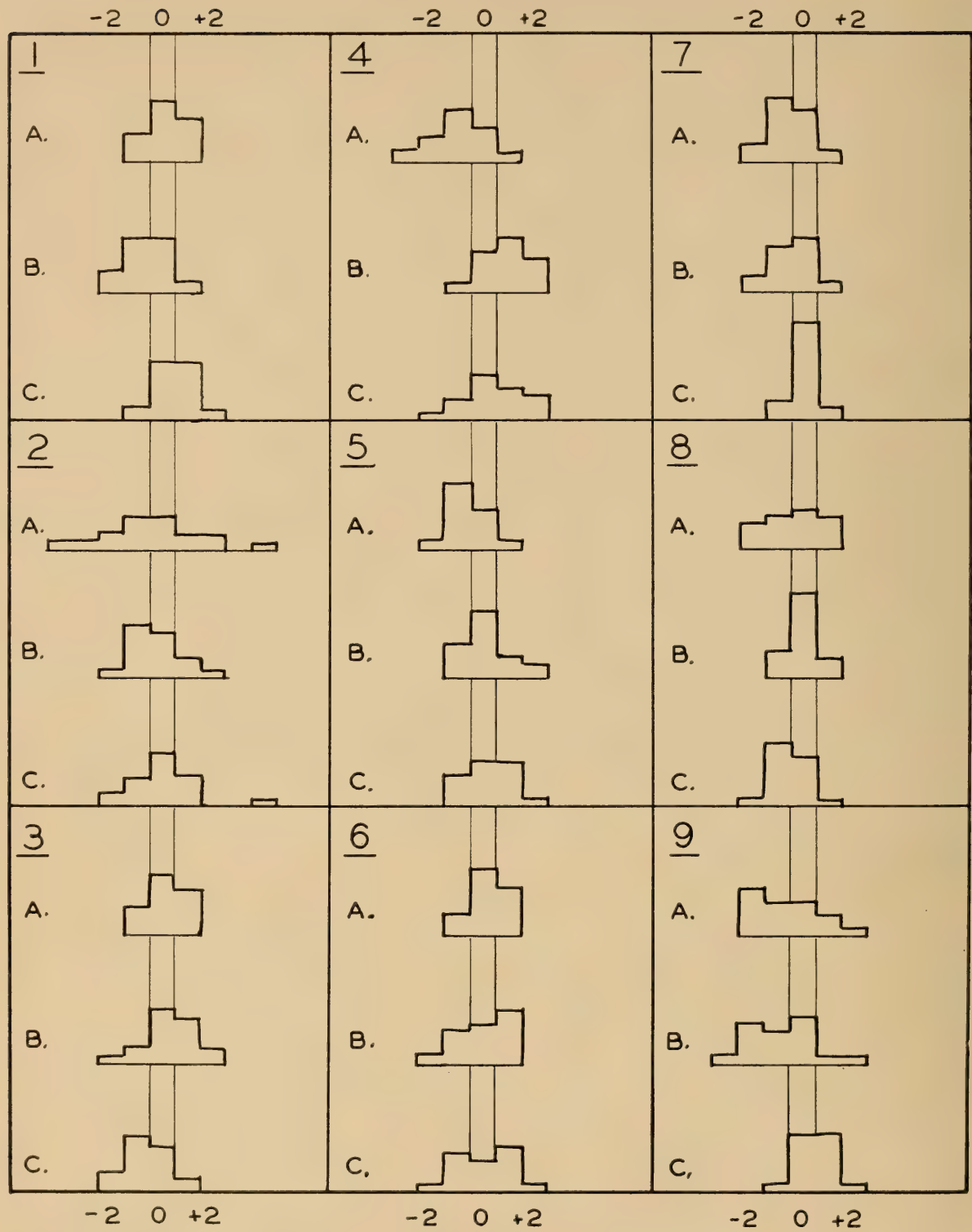


FIGURE 3. The frequency distributions of snail paths found in nine sample series of 60 runs. A, 20 runs in E-W magnet field. B, 20 runs in N-S magnet field. C, 20 control runs only in the earth's (N-S) field. See text for discussion.

4, 8) of the controls. The N-S oriented magnet could appear to effect more dispersion from zero than the controls within the same series (*e.g.*, #3, 7, 9) or less (*e.g.*, #2, 4, 8); it could appear to effect mean paths to right (*e.g.*, #4, 5) or the left (*e.g.*, #1) of the E-W oriented magnetic field (A in figure) or to produce more (*e.g.*, #3, 6) or less (*e.g.*, #2, 8) dispersion than that observed within the same series for the E-W oriented magnet. Comparably, the E-W oriented magnet was associated with patterns to left (*e.g.*, #4, 5, 7, 9) or right (*e.g.*, #3, 8) or with more (*e.g.*, #2, 7, 8, 9) or less (*e.g.*, #3, 6) dispersion than the controls. Ordinary tests for the significance of differences between means, or between standard deviations in the frequency distributions among the three groups of 20 even within these single series, yielded, not uncommonly, probabilities ranging from the 5% to well less than 0.1% level. There was no suggestion that these differences could be explained on the basis of any continuous trend in behavior during the experimental period of a series. In fact, this last possibility was greatly reduced by having each sample of twenty composed of two groups of ten separated by intervening runs under other conditions.

It seemed quite apparent that if a *bona fide* response occurred to either the earth's magnetic field or the experimentally imposed ones, the response could not be simple and invariable. Consequently, analyses of the data involved chiefly three types of values: (a) mean direction and degree of turning; (b) total dispersion as a measure of right and left turning about the zero axis; and (c) standard deviation as a measure of dispersion relative to whatever was the mean path of the given series. The forms of frequency distributions were also always inspected.

(A) *Mean path of snails*: Using all data, it became quickly evident that there was a daily rhythm in the average degree of turning of the emerging snails, the animals moving nearly straight ahead at 5 AM and turning increasingly to the left until noon. Thereafter, they turned progressively less, to a second minimum of turning about 7 PM. The mean paths for the snails exposed to the N-S and E-W experimental magnetic fields, and for the controls are plotted as a function of hour of the day in Figure 4A. Indicated also are standard errors of the means for the combined three groups for selected hours. In Figure 4B are illustrated the differences, hour by hour, between the path of the controls and the snails in each of the two experimental magnetic fields. Using all 1128 available differences between experimental and control animals, a mean of -0.0341 ± 0.0104 was obtained. This indicated that the presence of a magnet results in increased left-turning over controls ($P < .005$).

There was no statistically significant difference between the effect of the N-S field (-0.0347) and E-W one (-0.0336). It is, however, suggestive that the 3.2% greater effect of the N-S field is correlated with the actual 12% greater horizontal intensity of the experimental N-S over the experimental E-W field, due to the vectorially additive effect of the earth's natural field in the former experimental condition.

In view of the evident daily rhythm in the mean path observed, together with the suggestion from Figure 4B that increase in strength of magnetic field in the early morning hours yields greater *right* turning, an examination of the effect of the magnetic fields for the hours 7 AM through 9 PM was made. This gave even more decisive indication of increased left-turning in response to the experimental

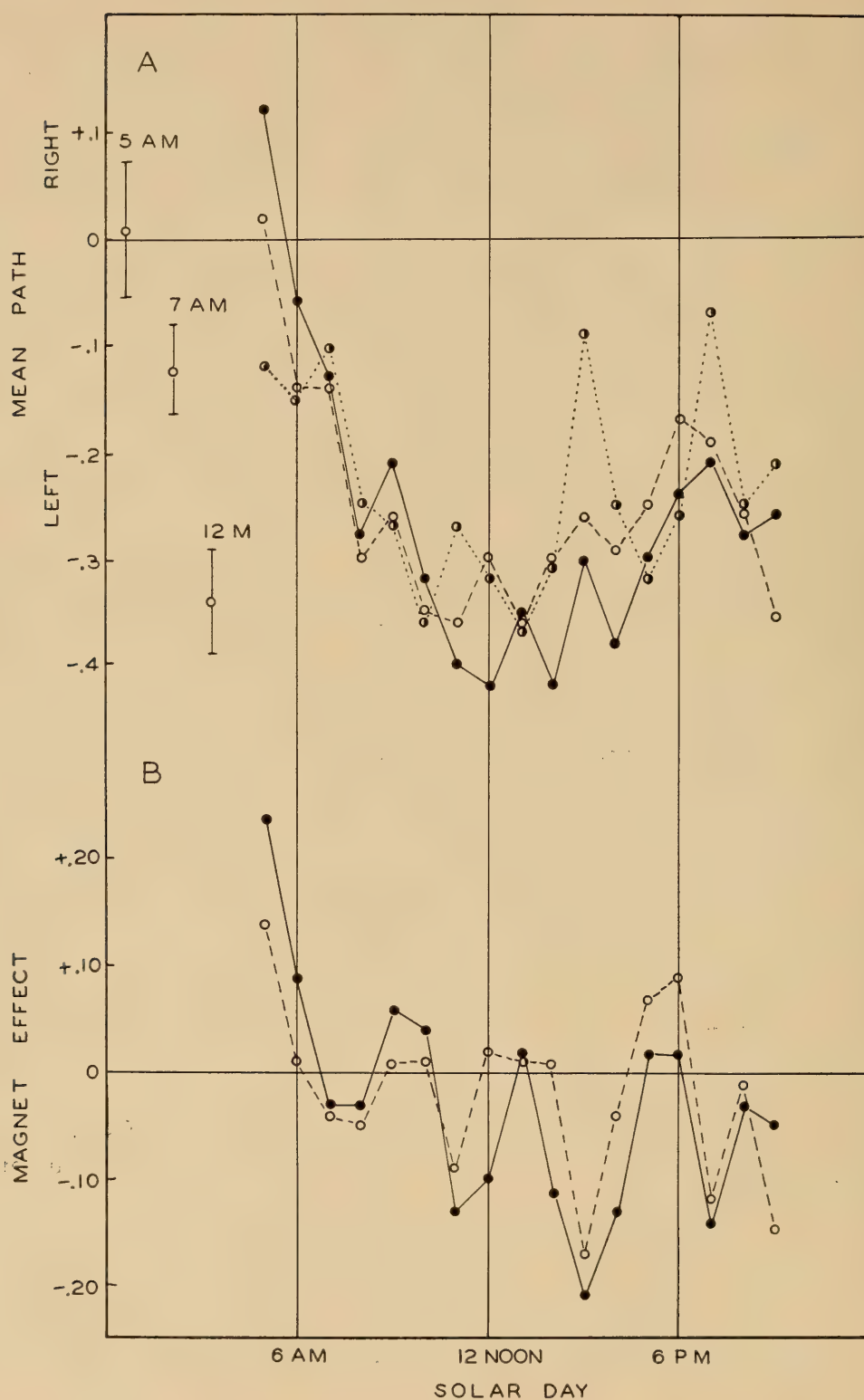


FIGURE 4. A. Mean paths of the snails in the E-W magnet field (dashed lines), in the N-S magnet field (solid line), and control snails (dotted lines) as a function of hour of the solar day. Standard errors of means for all snails are shown for three times of day. B. The difference of mean paths of snails in the E-W magnet field (dashed line) and N-S magnet field (solid line) from the paths of the control snails in the same series.

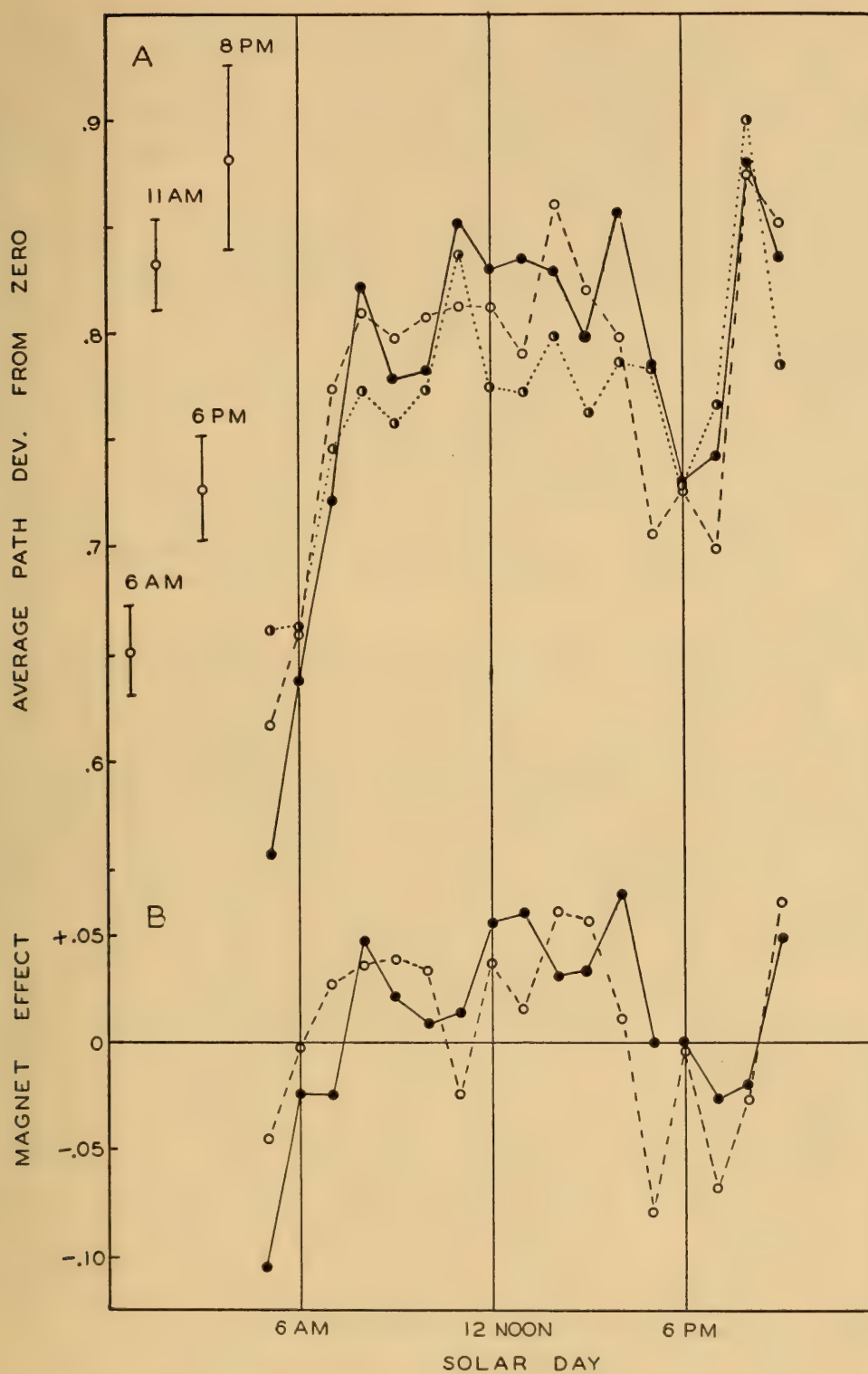


FIGURE 5. A. Mean dispersion of experimental and control snail paths from the zero sector as a function of hour of day. Standard errors of means from all snails are shown for four times of day. B. Difference between experimental snails and controls as a function of time of day. See Figure 4 legend for key.

magnetic fields ($P < .001$). Also, for the 3 PM hour alone the effect of the increased magnetic field strength was highly significantly different from zero ($t = 3.64$; $N = 84$). These probabilities, which leave no reasonable doubt as to a response to the magnet, are found despite highly significant fluctuations of magnetic response of lunar-day frequency which contribute greatly to the variability of the response (Brown, Webb and Brett, 1960).

(B) *Mean dispersion of paths from zero*: Inspection of the frequency distributions of the mean snail paths as a function of hours of the day, indicated an increasing bimodality of the distribution of the paths, through a maximum degree of bimodality near noon. A consequence of this is clearly apparent in the form of a daily fluctuation in average dispersion (Fig. 5A). Standard errors for selected hours are shown. A broad maximum is centered over noon, and a second maximum occurs at night. There is also evident a daily rhythm in the difference between mean dispersions of control and experimental animals (Fig. 5B). For a 9-hour period centered at noon, the mean dispersion of the animals as the average for the two magnetic fields was $7.55 \pm 1.96\%$ ($N = 364$) greater than that of the controls. These results suggest, further, that the effect of both magnet positions in early morning and early evening is to reduce dispersion even below that of the controls within the same series.

(C) *Standard deviation of paths*: The next analysis was of the fluctuations in standard deviation of paths. It was clear that there was a daily fluctuation in this parameter. This is illustrated in Figure 6A. The significance of this cycle can be seen from the two standard errors included. In this parameter, as in the others, the frequency distributions suggested increasing bimodality for the data obtained over the middle of the day. However, at those times of day, 8–12 AM and 8 and 9 PM, when standard deviations are relatively lower than might be expected in terms of total dispersion (compare Figs. 5A and 6A), the population of values more strongly favors one of the two centers in the bimodal frequency distribution. Figure 6B illustrates the hour-by-hour difference between the control snails and the snails in the experimentally augmented magnetic fields. The daily pattern of effect of the imposed magnetic fields upon altering the standard deviation of paths selected appears to possess at least three maxima. With all the data from the 17 hours of the day, the standard deviation appeared increased by the magnets, though not highly significantly ($P < .05$). For the period 6 AM through 4 PM alone, the statistical significance increased substantially ($P < .01$). However, that an influence of the magnetic fields is being reflected in this parameter is suggested even more strongly from the similarity of the mean daily pattern of effect for the two magnetic orientations, N–S and E–W ($r = 0.72$) ($N = 17$), though the significance of this correlation is obviously tempered somewhat by the fact that there was a common control group for the two experimental groups.

(D) *Frequency distribution of magnet responses, relative to hours of day*: In view of the clear indication from the preceding analyses that the imposed magnetic fields effect to various extents either of two types of response, right or left turning, it seems reasonable to presume that (1) the small, but statistically highly significant, predominance of left turning especially between the hours 7 AM and 9 PM, and (2) the clear suggestion of right turning at 5 and 6 AM are simply residuals, a consequence of failure of one response to be cancelled exactly by the

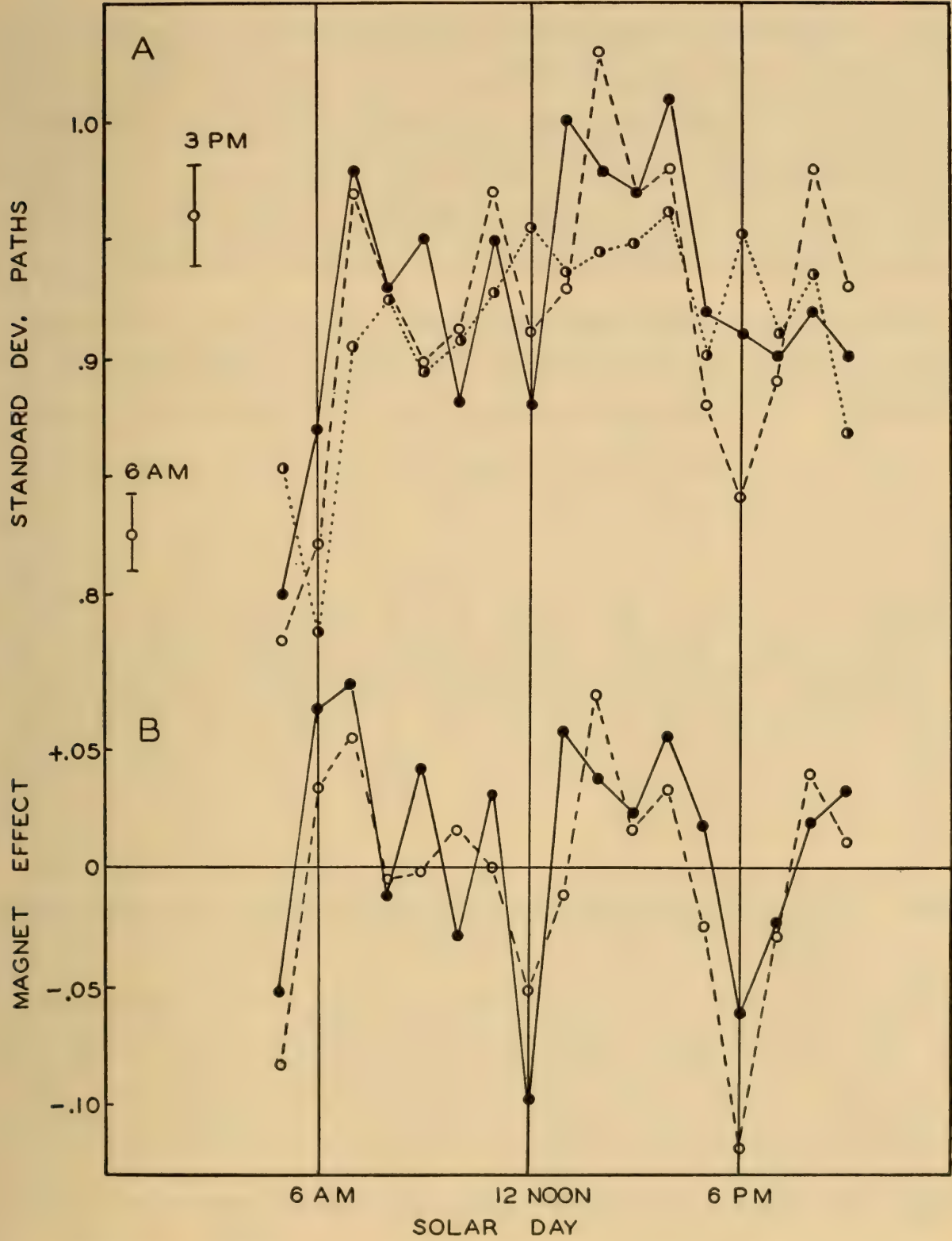


FIGURE 6. A. Standard deviation of experimental and control snail paths, as a function of hour of day. B. Difference between experimental snails and controls as a function of time of day. See Figure 4 legend for key.

other. To cast further light upon this aspect of the problem, frequency distributions of the differences obtained between experimental animals and the controls in the same series are shown as a function of hour of the solar day in Figure 7. From the general dissimilarity of nearly all these to normal distributions, and the strong tendency towards bimodal distributions (see especially 9 AM through 4 PM), these suggest that the organismic response to the imposed magnetic fields is not only real, but substantially larger than was apparent from the simple study of the pooled hourly data. The actual response appears normally to amount to magnetic-induced turning through an average of 0.3 to 0.4, or more, of a sector unit, or a mean turning of the order of $8-10^\circ$ during the snail passage in a few seconds over the 3-cm. course.

Further inspection of the distributions suggests that a response to the imposed magnet occurs also as a deviation from a mean degree of turning characteristic of the particular hour of the solar day. This is most evident in the 3 PM and 9 PM distributions. It is also suggested for the 5 and 6 AM hours, when the mean

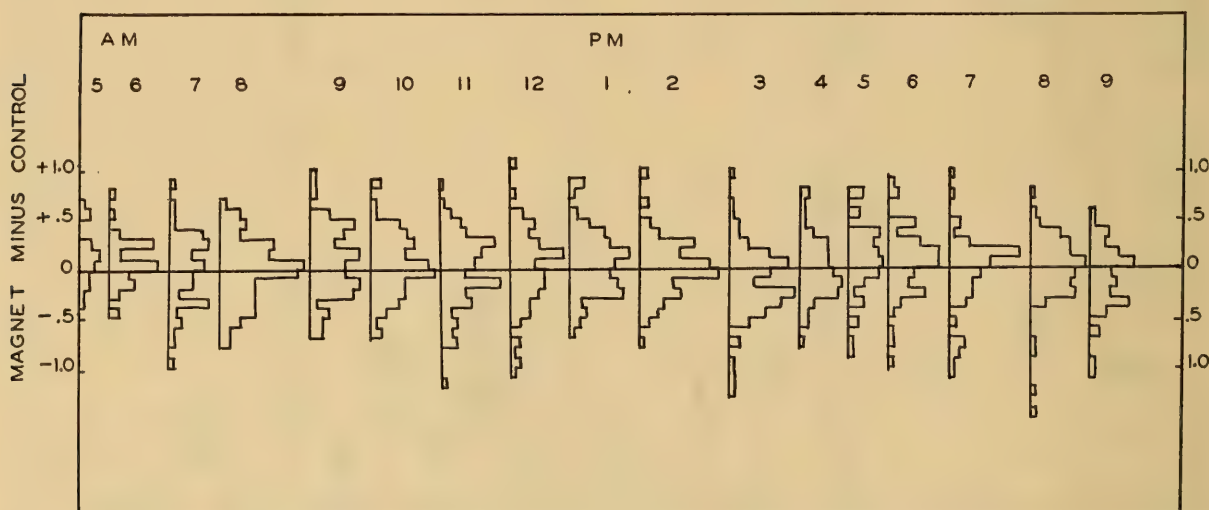


FIGURE 7. Forms of frequency distributions of the differences between all experimental snail paths and control snail paths, as a function of hour of the solar day.

turning of controls is near zero and the response to the magnet is predominantly that of turning to the right. It appears also evident that the mean effectiveness of the two experimental magnetic fields passes through minima at 8 AM, 2 PM, and 7 to 8 PM, as indicated by the reduction in the bimodality at these times. This changing pattern of frequency distributions through the day suggests the presence of two major daily patterns of oscillation in the extent of the response to the experimentally increased magnetic fields, with one pattern essentially the mirror-image of the other, and with cross-over points at these times of minimal response.

DISCUSSION

It is apparent from this study that in the constant symmetrical experimental field the amount of turning of snails is a function of the time of day, and that a daily fluctuation is found in both the mean amount of turning, ignoring sign, and

in the mean path of the snails taking sign into account. This is apparent for all snails, both those in the earth's natural magnetic field and in the imposed fields where the field strength is increased about 10-fold with the direction of magnetic lines of force being left either in the same direction as the earth's, or at right angles to this. The three groups considered as three samples showed remarkable mean similarities as functions of hours of the day, though commonly differing very widely from one another in any individual series (see Fig. 3). This similarity indicated that the amount of right or left turning, or dispersion of paths from zero, was not determined primarily by the direction of the field or by its fluctuations in strength.

Hence, it must be concluded that the response is predominantly a klinokinesis, or a turning relative to any initial compass direction, either clockwise or counter-clockwise, as a function of time of day. Of the various possibilities of the factors concerned in the response, three are immediately apparent. (1) The response involves a rate of turning relative to the diffusing light source above and slightly ahead of the path of the snail. This might be interpreted as a move on the part of the snail to assume a particular angular relationship between the long-axis of its body (light-compass relationship) and the artificial gigantic "sun" as a function of the time of solar day; (2) the turning of snails involves a torsional orientation in response to the magnetic field itself as a function of time of day; (3) the orientational response may not be due to any external factor directly, but simply to differential bilateral activity of bodily orientation mechanisms. Or, possibly any two or all might be involved and contribute jointly to the results. However, it is evident from this study that just as would be expected were the response normally one of klinokinesis in response to the magnetic-field, so the klinokinesis can be highly significantly modified through experimentally increased magnetic flux as a function of time of solar day. There also remains an additional possibility, namely, that any light-compass orientation of the snails is, in turn, regulated through an orientation to magnetic field.

Support for the concept that the normal turning of the snail is importantly an orientation in response to magnetic field itself may be seen first in Figure 5. When the dispersion is least in the earth's magnetic field, the effect of increasing magnetic flux is to reduce it still more, and when dispersion is large in the earth's field, the magnet increases it still more. Comparably, as Figure 4 indicates, when left-turning is high in the earth's natural field the magnet increases it, and when it is low, appears to effect turning in the opposite direction, to the right. In a general manner, but slightly more complexly, the relationships of Figure 6 also give a similar kind of support for the view of an important role of the normal earth's field in snail orientation. The effect of the experimentally augmented field is to bring about either further increased, or decreased, size of standard deviation, depending upon which is normally the predominant response characteristic of the time in the daily cycle.

It is very interesting, also, to compare the two aspects of magnetic orientation, left-turning and bimodality of distribution about a mean path, to the two signs of oxidative metabolic fluctuations of *Nassarius* about a mean daily non-inverting fluctuation. Both the non-inverting and inverting components in these metabolic fluctuations appear clearly to be responses to unidentified barometric pressure

correlates (Brown, Webb and Brett, 1959). This suggests an intimate relationship between the mechanisms of temporal and spatial orientation in the snails and points to the possibility that the experimentally augmented magnetic field has in part simply increased the strength of whatever orientational physiological mechanism chanced to be dominant at the time, through a generalized influence upon the mechanism of cellular oxidative energy transformations. For this effect, the magnetic field would need to possess no spatial orientational feature, *per se*. But while this effect might account for the induced increases in standard deviation or increased turning, it could scarcely account for those times of improved precision of orientation by reduction of turning below that of the controls. The last appears more rationally accountable in terms of the concept that the sharp and stronger the magnetic field, the more decisive whatever organismic responses are characteristic of that time of day to it.

SUMMARY

1. The orientation of snails in a constant, symmetrical field was studied over a two-month period, June 28 through August 29, 1959, at various hours of the day between 5 AM and 9 PM.

2. The orientation of snails in the earth's natural magnetic field was compared throughout the study with the orientation of snails subjected to a 9- to 10-fold increase in field strength, with fields both parallel and at right angles to the earth's natural field.

3. A daily rhythm in the direction and average amount of turning was found in the snails; the mean paths of those in the two (N-S; E-W) experimentally augmented magnetic fields were statistically significantly to the left of the controls, particularly between the hours 7 AM through 9 PM.

4. The mean amount of turning, whether clockwise or counterclockwise (klinokinesis), in the experimental magnetic field was also increased significantly over that of controls in solely the earth's field, and similarly exhibited a daily rhythm.

5. Certain similarities between the orientational responses to the magnetic fields and earlier described exogenous metabolic fluctuations in constant conditions, suggest a relationship between them.

6. Evidence is advanced supporting the hypothesis that the orientation of snails normally includes a true response to the earth's magnetic field.

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MAGNETIC RESPONSE OF AN ORGANISM AND ITS LUNAR RELATIONSHIPS¹

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It has been demonstrated (Brown, Brett, Bennett and Barnwell, 1960) that mud-snails, *Nassarius obsoleta*, initially directed magnetic southward in a symmetrical field constant for all factors normally considered able to influence their orientation, exhibit a daily rhythm in the direction of their mean paths. The amount of dispersion of paths of a population of snails about their mean path also displays a daily rhythm. Both of these characteristics of spatial orientation were shown to be quantitatively alterable, in a manner highly significant statistically, by experimentally changing the strength of the ambient magnetic field. Although much of the observed variation, both in the orientation of control animals in the earth's field and in the modified orientation in response to experimentally increased strengths of the magnetic field, was accounted for in terms of the daily rhythm of response, much variation still remained unaccounted for. The following study was made to determine whether lunar periodisms in responsiveness to the magnetic field were also present.

METHODS AND MATERIALS

The apparatus, and methods of obtaining the data, utilized in this study have already been reported in detail elsewhere (Brown, Brett, Bennett and Barnwell, 1960). In essence, measurements were made of the average amount of clockwise or counter-clockwise turning of snails as they traversed a 3-cm. course immediately following their emergence from a straight, narrow corridor directed toward the magnetic south into an illuminated, symmetrical, constant field. Each of 564 experimental series, obtained during the period June 28 through August 29, 1959, included two groups of exits of ten snails each in the earth's natural magnetic field, two groups of ten in a magnetic field increased 10-fold and oriented as the natural one (N-S), and two groups of ten in a nearly equally strengthened field rotated clockwise through 90° so that the north-seeking pole of the bar magnet was directed west (E-W).

Data were obtained for hours of the day from 5 AM through 9 PM, E.S.T., with no hour of this period represented by fewer than nine series, nor more than 50 series, of 60 snail exits.

RESULTS

Lunar-day rhythm of mean path

All the data on orientation of the snails for each calendar day were rearranged to assume their proper relationship as approximate hours of lunar days. When

¹ This research was aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, #1228-03.

more than one series was recorded for any single hour of a given day, these were combined to yield a single average value. The average direction and amount of turning of the snails as a function of hour of the lunar day is indicated in Figure 1 for the control snails, those in the artificially augmented N-S magnetic field and those in the E-W field. Each of the three differently treated groups is dealt with here as a separate sample. Not only is there a clear mean lunar-day cycle of

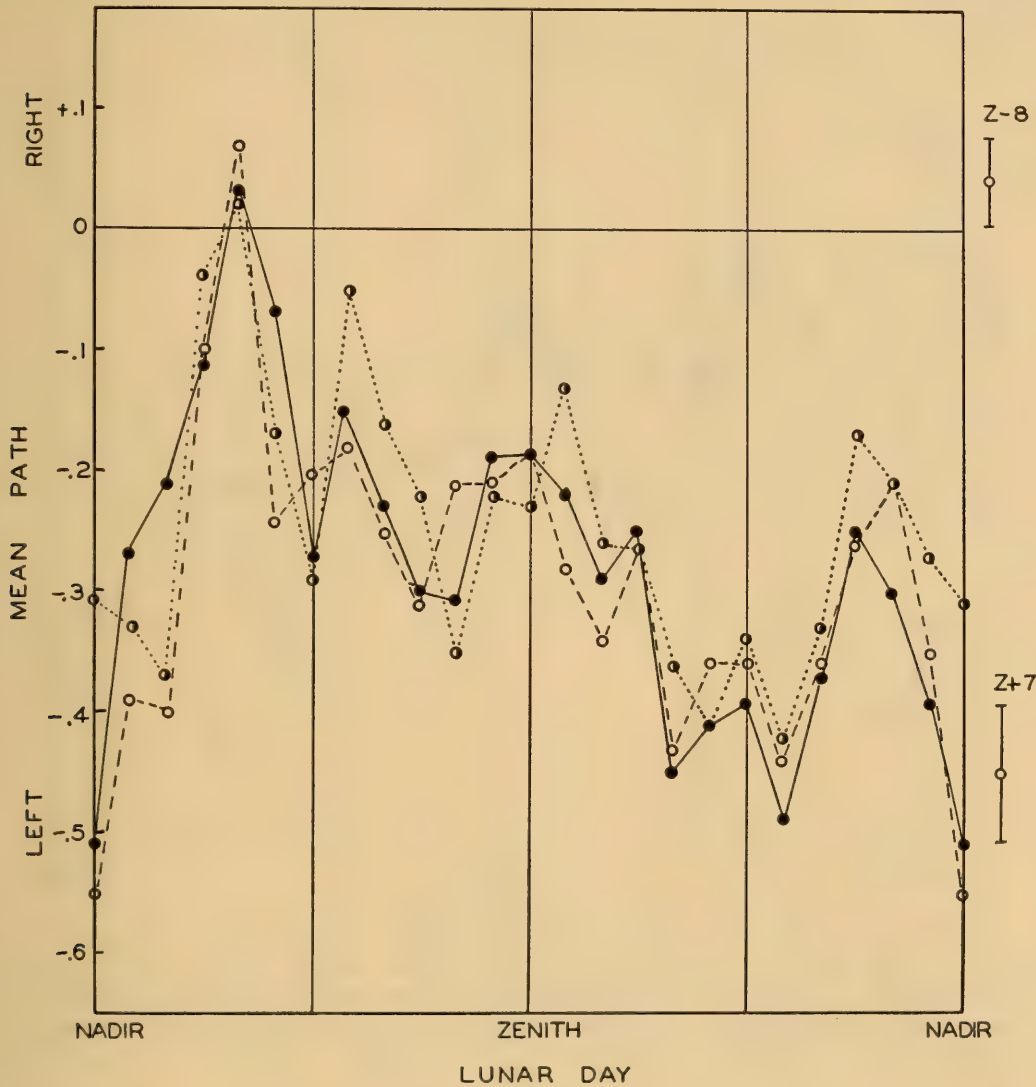


FIGURE 1. The mean paths of snails in the E-W magnetic field (broken line), N-S magnetic field (solid line), and control snails solely in the earth's field (dotted line) as a function of hours of the lunar day. Standard errors of means at two selected times are depicted.

turning, but the three samples exhibit a considerable similarity in the form of the mean cycle despite relatively wide differences between values for the three samples within individual series of 60 (See Brown, Brett, Bennett and Barnwell, 1960).

Not only is the mean lunar-day cycle of turning of essentially the same amplitude as the comparable, previously described solar-day one, but displays a general similarity to it in gross form, though appearing to mirror-image the daily one in

secondary superimposed fluctuations over the period the moon is above the horizon. As with the solar-day cycle, minimum left-turning or maximum right-turning occurs about the fourth hour of the lunar day or near the time of moon-rise, and maximum left-turning occurs at lunar nadir. Standard errors of the means for the fourth (zenith minus 8) and the nineteenth hours (zenith plus 7), calculated from all data for these hours, are indicated on the figure. These values indicate the high degree of statistical significance of this lunar-day fluctuation of mean path of orientation.

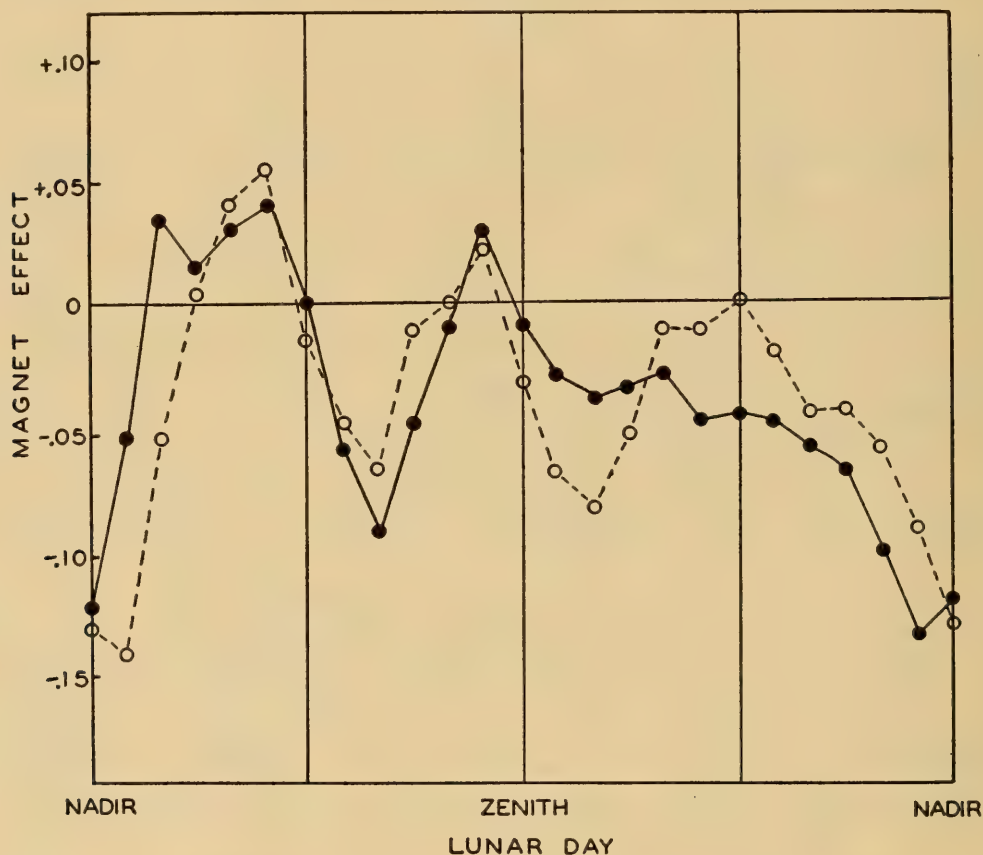


FIGURE 2. The difference of mean paths between snails in the E-W magnetic field (broken line) and N-S magnetic field (solid line) from control snails as a function of hours of the lunar day. The values are calculated from all series studied during overlapping three-hour periods.

Lunar rhythm in effect of experimentally augmented magnetic fields

The average effect of the imposed magnetic fields for overlapping groups of three lunar-day hours were expressed as differences from the comparable three-hour control samples and the mean lunar-day fluctuation in this magnetic effect is plotted in Figure 2. Such three-hour grouping of the data was employed (1) to reduce as far as possible the apparent significance of individual points based upon relatively small numbers of series, and (2) to reduce irregularities in the lunar-day cycle reflecting incomplete randomization of the solar-day cycle as a consequence of the occurrence of occasional solar days lacking in data. As indicated in Figure 2, a maximum right-turning response to the experimental magnetic fields occurs near

the time of moon-rise, and a maximum left-turning response is seen at lunar nadir. This is true whether one deals with either the N-S or E-W experimental fields. The lunar-day pattern suggests three progressively decreasing maxima as one moves through the lunar day. The overwhelming preponderance of negative values, indicating the induction of left-turning by the experimental procedure, clearly corroborates the results of the earlier solar-day study in demonstrating this response to the experimentally imposed weak fields.

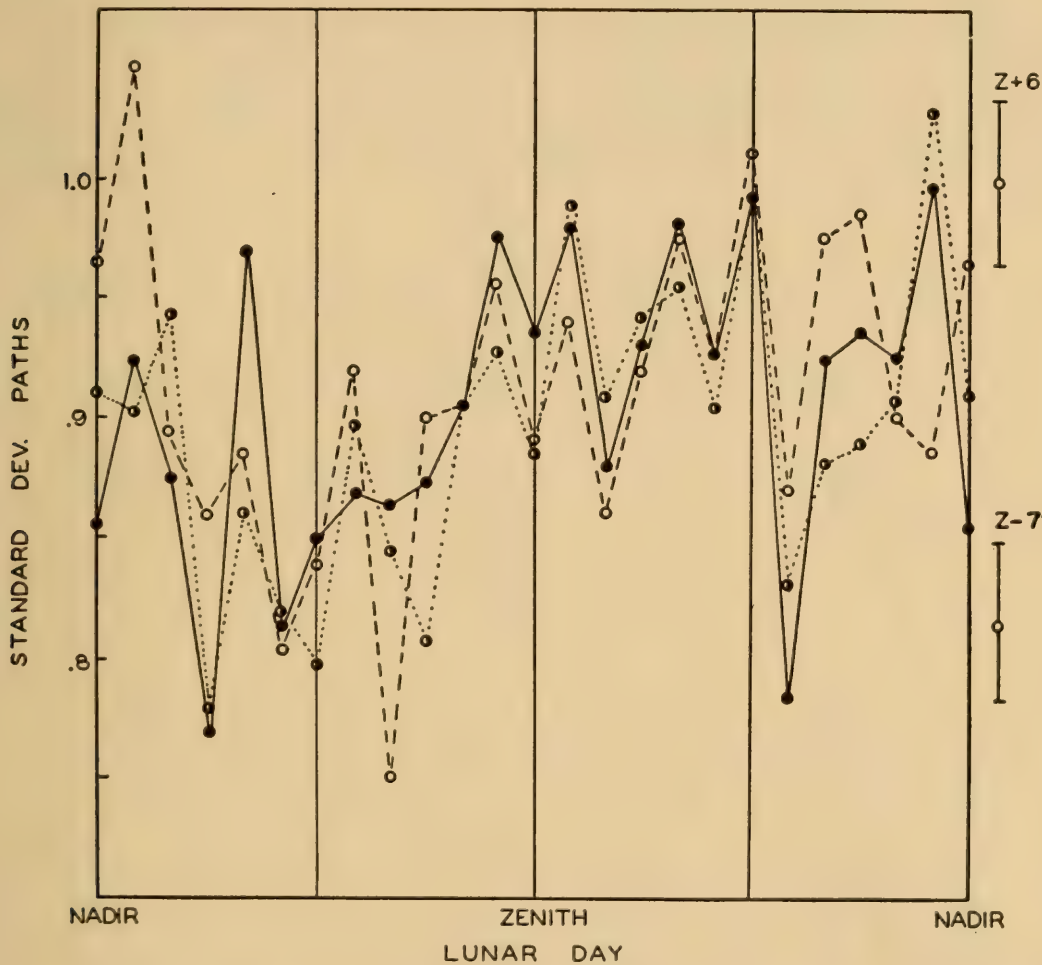


FIGURE 3. The standard deviation of the paths of experimental and control snails as a function of hour of lunar day. Sample standard errors are illustrated. See legend of Figure 1 for key.

Standard deviation of paths

In view of the solar-day rhythm of dispersion of paths established previously and indicating both right and left turning responses to the magnetic field, the standard deviations of all the samples of 20 controls, 20 animals in the N-S experimental field and 20 in the E-W field were next rearranged to ascertain whether a lunar-day fluctuation occurred also in this parameter. In Figure 3 is seen the mean lunar-day fluctuation of standard deviations for the month-period, July 6–August 4, for each of the two experimental conditions and the controls, separately. Standard errors of the means for the seventh hour before zenith and the sixth hour

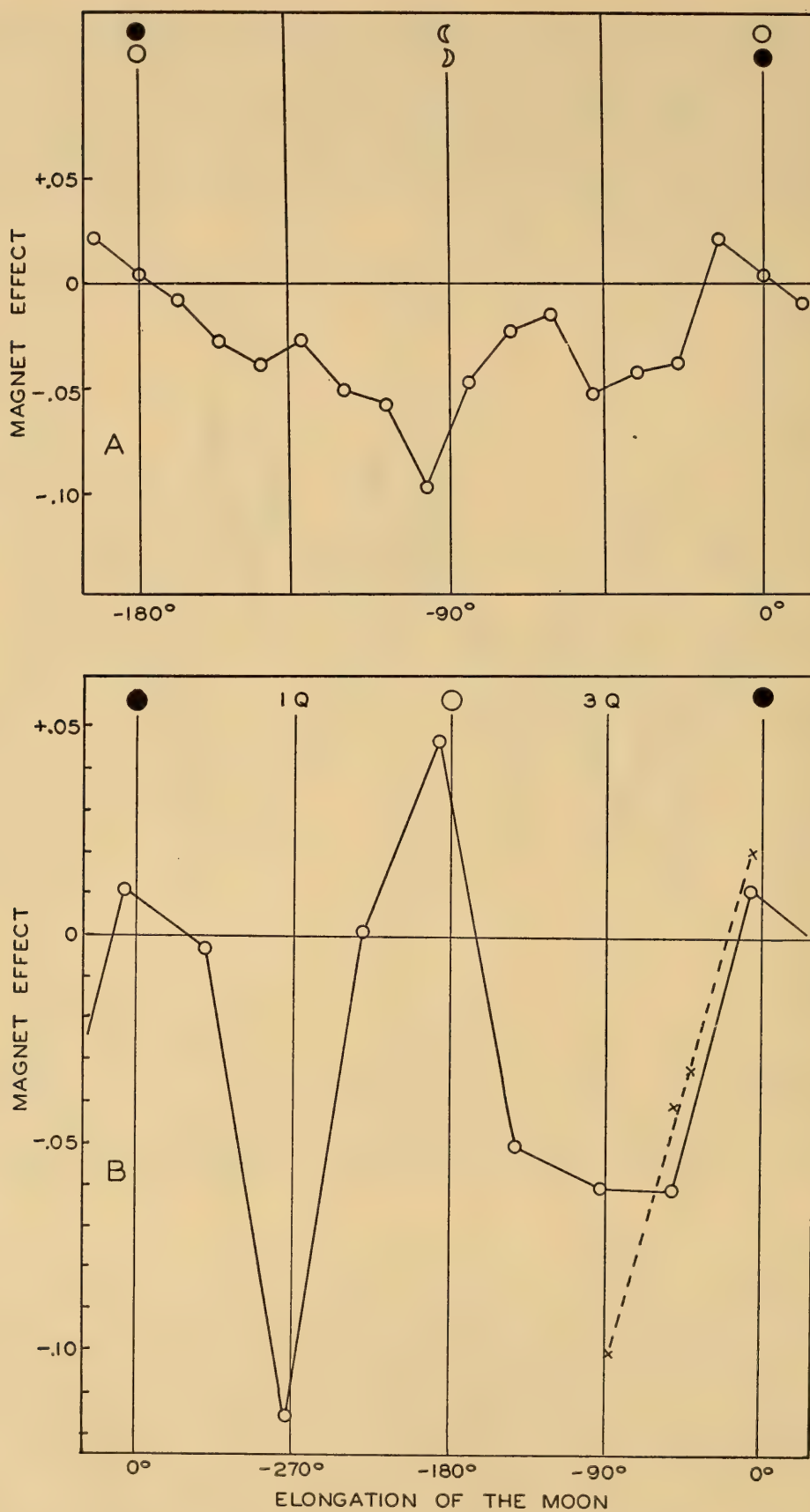


FIGURE 4. A-B.

after zenith indicate the highly significant character of this lunar-day cycle. When, instead, all data for the two-month period were used, this cycle was very similar, but had slightly increased amplitude and statistical significance. However, the particular monthly period was selected since the most complete series of daily studies were conducted during this period, and, hence, more complete randomization of the solar-daily cycle was assured.

Semi-monthly cycle in the mean daily sign and amount of turning

A semi-monthly rhythm of response to the experimental magnetic fields was noted. This was characterized by the experimental snails turning to the right of controls just before each new and full moon, and turning maximally to the left of controls near the times of the moon's quarters. Differences between the paths of experimental snails in the N-S or E-W fields and controls were obtained for a

TABLE I

Mean daily signs of magnetic field responses obtained as a function of day of a natural semi-monthly period

NM or FM	+	+	+	-		
+1	-	-				
+2	+	-	-	-	-	-
+3	+	-	-	-	-	-
+4	0	-	-	-		
+5	+	+				
+6	+	-	-	-		
+7	0	-	-	-		
+8	+	+	-	-	-	-
+9	-	-	-	-		
+10	+	+	-	-		
+11	+	+	-	-	-	-
+12	+	+	-	-		
+13	+	+	+	+		
+14	+	+	+	+		

total of 64 days during the two-month period of study. The sign of the average response obtained for each of the days on which any series were obtained, as related to the days of new or full moon, is indicated in Table I. In view of the paucity, or absence, of data on some days and the relative abundance on other days it was felt that a more accurate determination of the mean form of this semi-monthly cycle could be obtained by calculating averages of all available series for overlapping periods of three consecutive days each. The results of such a procedure are plotted in Figure 4A.

The values were obtained by considering each day on a semi-monthly frame of

FIGURE 4. A. Semi-monthly cycle in mean daily difference between paths of experimental and control snails. The mean values from overlapping three-day periods of study comprise the points. B. The solid line indicates the fluctuation through the synodic month of the response to the experimental magnetic field based upon 3- to 5-day grouped data, selected to provide mean values at about 45° intervals of elongation of the moon relative to the sun. The broken line shows the relationship after averaging the original data for the two semi-monthly periods, considering both the times of new and full moon as 0° , and using the single range 0° to 90° .

reference of the scales new moon through new moon minus 14 days and full moon through full moon minus 14 days. This provided an arbitrary 15-day semi-monthly period, an adequately close approximation, for the purposes at hand, to the mean, natural 14.8-day one.

In order to assay the statistical significance of this semi-monthly cycle the correlation of the effect of the magnets with the elongation of the moon relative to the sun was assayed. The daily angle of elongation is 12.2° . However, inspection of Figure 4A suggested that the maxima and minima preceded the days of the new and full moon, or its quarters, by about half a day, or by about -6° . This value, consequently, was treated as the corrected zero one. The data were next combined into eight groups of days in the synodic month: (1) New moon, -1 , and -2 ; (2) -3 , -4 , -5 , -6 ; (3) -7 , -8 , -9 ; (4) -10 , -11 , -12 , -13 , -14 ; (5) Full moon -1 , -2 ; (6) -3 , -4 , -5 , -6 ; (7) -7 , -8 , -9 ; and (8) -10 , -11 , -12 , -13 , -14 . These times were selected to give values centered on times close to those in which the earth-moon and sun-moon axes were parallel with one another (0°), at right angles to one another (90°) and midway between these two (45°). The eight values are plotted in Figure 4B, now corrected for the -6° . When these were treated as linearly correlated with the angular elongation of the moon corrected to zero at -6° and one now remained with the 0 to -90° range of each quadrant of the monthly cycle of lunar elongation, a coefficient of correlation of 0.8486 ($P < .01$) was found. When the same data were further reduced by combining the original data for those pairs of values related to one another by 180° , r became extremely large, 0.9994 (broken line in Figure 4B). This last is obviously a very highly significant value. The substantially lower value of r with $N = 8$ than with $N = 4$, in this instance appeared to reflect a quite symmetrical difference between the general forms of the pre-new-moon and pre-full-moon semi-monthly cycles.

The response to experimental magnetic fields appears definitely correlated with the elongation of the moon, and in a rather regular manner. However, the restrictions in the number of values used in the reduced data clearly do not permit one to conclude with any certainty that the correlation is a truly linear one.

Synodic monthly fluctuation in mean daily standard deviation of paths

In Figure 5A are plotted together the mean daily standard deviations of paths of the controls, and the experimental snails in the N-S and E-W magnetic fields. Each is treated separately. During the first forty days of the study there appears to be a synodic monthly fluctuation in standard deviation, with the maximum deviation about two days prior to new moon and minimum deviation about the time of full moon. The last 13 days of data are not in any fundamental manner incompatible with the view of a continuing monthly periodism of the same general character, but do contain, over a short period, some tremendous fluctuations in the standard deviations which clearly can not themselves be part of a simple monthly cycle of the relative regularity of the first. This suggests significantly differing responses of the snails of an aperiodic character, in the constant conditions.

Figure 5B is a plot of the mean daily barometric pressure values for the corresponding period of time. The barometric pressure changes appear related more

than coincidentally to the fluctuations in standard deviations of the snail orientation. Not only are the snail orientation and the barometric pressure fluctuations the mirror-images of one another in gross general trends, but when the barometric pressure fluctuation is treated as a two-day lead correlation on snail orientation,

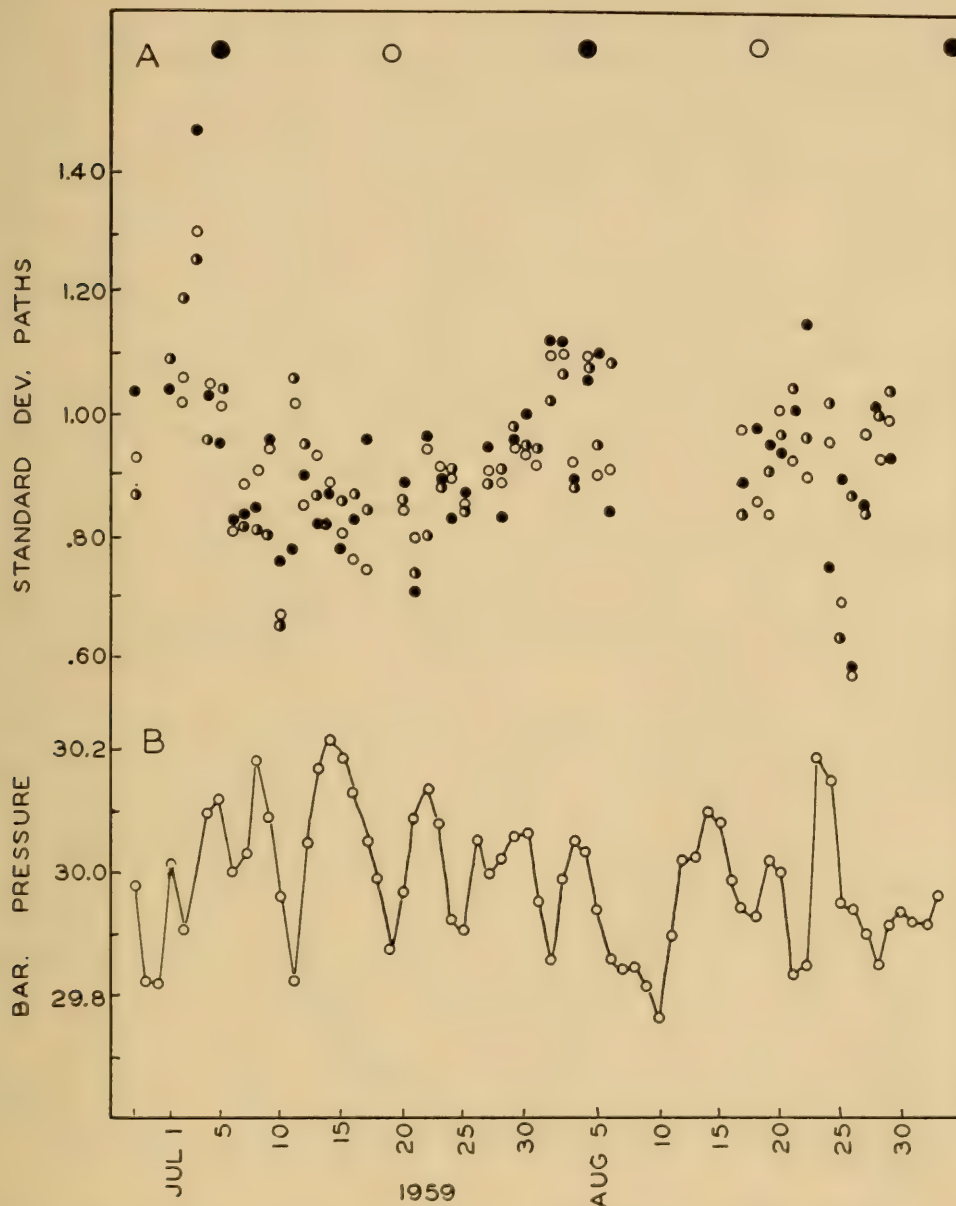


FIGURE 5. A. The mean daily standard deviations of all snail paths for the whole period of study. Open circles, snails in E-W magnetic field; closed circles, in N-S magnetic field; half open circles, controls. B. Mean daily barometric pressure for the period corresponding to (A).

even a number of the major irregularities in the fluctuations of snail orientations, and particularly those of the last thirteen days, become strikingly accounted for in these terms. The calculated two-day lead correlation was $r = 0.33 \pm 0.08$ ($N = 141$), clearly highly significantly different from zero.

DISCUSSION

There appears little reason to doubt that spatial orientation of snails, expressed as an amount of turning, or a klinokinesis, possesses a lunar rhythmicity. This is displayed first as lunar-daily, or approximately 24.8-hour, periodicities in both mean orientational paths with signs taken into account and total turning, both clockwise and counterclockwise. The latter is indicated here in terms of standard deviation of pathways. It is interesting that the form of the lunar-day cycle, in the range of hours corresponding roughly with those available for the solar-day study, namely 5 AM through 9 PM, is strikingly similar to that of the solar-day one in both gross features and phase relationships. At both the times of sun-rise and moon-rise the left turning is minimal and it generally increases towards the early solar and lunar "evenings,"—i.e., times of setting of sun or moon. Also, in both the solar and lunar days standard deviation of pathways is minimal about the time of sun-rise and moon-rise, and increases systematically while the sun and moon are above the horizon, decreasing again as these heavenly bodies set. Furthermore, the amplitudes of the solar and lunar cycles are of the same general magnitude. The presence of these two similarly conspicuous periodisms would be expected to give rise to a synodic monthly cycle, and such a cycle appears to be present. The occurrence of a semi-monthly cycle of influence of the experimental magnetic fields suggests that either the lunar-day or solar-day cycle of mean path is bimodal. Though the former is suggestive of bimodality, the complete form of the solar-daily cycle has not been determined. A semi-monthly rhythm would conform to that of the recurrence of the tides at a particular hour of the day in the natural habitat.

It is evident that since the snails were always subjected, whether in the field or laboratory, to daily illumination changes, a solar-daily cycle could be imparted to the snails by light cycles. The lunar-day period, on the other hand, could be gained by the snails in the field from the tides, but during the periods of as long as 8 to 10 days when the snails were retained in the laboratory any exogenous lunar period in terms of obvious environmental factors was not available. In view of the persistence of the lunar-day rhythm with conspicuousness equal to that of the solar-daily period, it would appear probable that both the lunar-day cycle and solar-day cycles are being derived continuously in response to some unidentified subtle environmental factors in a manner comparable to that demonstrated for the solar and lunar periodisms in *Uca pugnax* (Webb and Brown, 1958; Brown, 1959) and of the metabolism of *Nassarius* itself (Brown, Webb and Brett, 1959).

The similarities between barometric pressure changes and certain parameters of spatial orientation of snails suggest strongly that orientation in the snails is in some manner dependent upon subtle environmental factors other than magnetic fields and that these other factors may also induce considerable alteration in snail behavior. This is indicated, since even the experimentally augmented magnetic fields may be overridden by the factor correlated with the barometric pressure. One possible factor which has been suggested is electrostatic field (Webb, Brown and Brett, 1959). The correlation with barometric pressure recalls the correlation observed for metabolic fluctuations in the snails in constant conditions, including pressure, during the summer of 1958 (Brown, Webb and Brett, 1959), except

for its being a mirror-image. Since there is reason to believe (Brown, Brett, Bennett and Barnwell, 1960) that the spatial orientation described in this study is at least in some measure a klinokinetic response to the natural and experimental magnetic fields, it is possible that fluctuations in other subtle geophysical factors can influence the sensitivity of the magnetic responder-system. However, the possibility can not be excluded that a responder system for another factor is being altered by the magnetic field changes. The apparent effectiveness of certain barometric pressure correlates in modifying the orientational responses, and the well-established role of these correlates in influencing the rate of cellular oxidations, suggest that the sensitivity to the magnetic field is in some manner related to rate of oxidative metabolic changes.

These striking relationships between strength and character of magnetic orientation and phases of the lunar-day and synodic monthly periods contribute, together with the earlier demonstrated solar-day relationships, a substantial degree of predictability to the responses of the snail to magnetic forces. Collectively they increase still more not only the probability that this magnetic response is real, but that it plays a significant role in both the lunar-dominated tidal and the solar-day periodic behavior of these animals.

It is interesting, further, to note that in the lunar-day fluctuation in orientation, with sign taken into account, those times of lunar day when the mean path of the snails is essentially straight or even slightly to the right, the experimentally augmented magnetic field effects turning to the right, and as the snails normally orient during the lunar day more and more to the left as one approaches lunar nadir, the effect of the increased magnetic field is to produce progressively stronger left-turning. This relationship is quite comparable to that found for the solar day and provides further support for the view that response to the earth's natural field normally occurs.

There has been noted a remarkable similarity in both the gross form and the detailed trimodal character of progressively decreasing maxima of the lunar-day, N-S magnetic field responses of snails and the mirror-image of a highly significant mean lunar-day cycle of spontaneous motor activity found in white mice over the 5-month, partially overlapping period, March through July, 1959 (Terracini and Brown, unpublished) similarly treated as a three-hour moving mean. The maxima and minima were essentially synchronous when the two cycles, studied in two places, Massachusetts and Illinois, were adjusted to simultaneity (universal time). The coefficient of correlation for the two trimodal cycles was -0.85 . Similarly high simultaneous correlations have been reported earlier between fluctuations of the nucleonic component of cosmic radiation in Illinois and fiddler crab and sea-weed metabolism in constant conditions in Massachusetts (Brown, Webb and Bennett, 1958). This similarity between the cycles of mice activity and response of snails to the magnetic field supports the hypothesis that in this magnetic response we are dealing with a widely, probably universally, occurring biological phenomenon.

The proof of a remarkable sensitivity of an organism to one subtle environmental factor (magnetic field) operating at an intensity only slightly above the earth's natural one, and the evidence for its modification in these constant conditions by fluctuations in a second natural factor, poses even the very fundamental problem

of the dispensability, or indispensability, of the earth's milieu of subtle factors and its natural fluctuations, as a normal ecological consideration in organismic survival and species propagation.

SUMMARY

1. The direction, and mean amount, of turning in snails initially directed southward into a constant symmetrical, illuminated field displays a lunar-day rhythm with minimum turning about the time of moon-rise, and maximum turning at lunar nadir. There is also a lunar-day cycle of standard deviation of snail pathways, with a minimum about moon-rise and a maximum near moon-set.

2. The response of snails to an experimentally augmented magnetic field also exhibits a lunar-day rhythm with maximum turning to the left at lunar nadir.

3. The specific character of the lunar-day rhythm of the response to the experimental magnetic fields gives further support for the view that magnetic field is normally involved in snail orientation.

4. The mean daily response of snails to experimental magnetic fields, expressed as differences from the response of controls in the earth's natural field, displays a semi-monthly rhythm. Maximum right-turning in response to a magnetic increase of 10-fold over that of the earth occurs one to two days before new and full moon, and maximum left turning just before the times of the first and third quarters of the moon.

5. There is a synodic monthly fluctuation in mean daily standard deviations of snail paths with maximum deviation about two days before new moon and minimum deviation about the time of full moon.

6. Some suggestive correlations are demonstrated between barometric pressure and the spatial orientation of snails in an environment constant with respect to all generally accepted orienting factors.

7. It is pointed out that similarities in influence of some unidentified barometric pressure correlates on (a) magnetic orientation of snails, (b) general cellular oxidations, and (c) spontaneous activity cycles present reasons for postulating that the latter two phenomena are in some manner related to the magnetic field response and suggest that response to magnetic field is a widely distributed biological phenomenon.

8. Evidence is presented that suggests there is a biological influence of a universal-time-related, rhythmic, environmental factor.

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INVESTIGATION OF THE HORMONES CONTROLLING THE DISTAL RETINAL PIGMENT OF THE PRAWN *PALAEMONETES*¹

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The distal retinal pigment of the prawns *Palaemon* and *Palaemonetes* has been observed after appropriate stimulation in the fully light-adapted position and the fully dark-adapted one as well as in an intermediate state (Kleinholz and Knowles, 1938; Sandeen and Brown, 1952). The position of the distal pigment was a function of the brightness of the visual field.

Migration of the distal pigment is mediated by blood-borne substances. Kleinholz (1936) found that extracts of eyestalks from *Palaemonetes* caused this pigment to migrate toward the fully light-adapted position. Brown, Hines and Fingerman (1952) obtained the same response with extracts of supraesophageal ganglia. The latter investigators also provided indirect evidence for a hormone that would move the distal retinal pigment toward the fully dark-adapted position. Direct evidence for a dark-adapting hormone in *Palaemonetes* was supplied by Fingerman, Lowe and Sundararaj (1959). When extracts of eyestalks were injected into prawns whose distal pigment was in a position approximately midway between the fully light-adapted and dark-adapted states, a light-adaptational response occurred and lasted about two hours. A dark-adaptational response that lasted approximately five hours followed the light-adaptational one. The supraesophageal ganglia appeared to contain light-adapting hormone alone. No dark-adaptational response was obtained with extracts of eyestalks that was not preceded by a light-adaptational one.

The major objectives of this investigation carried out on *Palaemonetes* were two-fold. The first aim was to learn if the amount of light-adapting hormone in the supraesophageal ganglia could be altered by maintaining specimens in light and in darkness. This aspect was part of a continuing investigation in our laboratory of the effects of long-term adaptation upon endocrine sources in crustaceans. The second objective was to effect at least a partial separation of the distal retinal pigment light-adapting and dark-adapting hormones from each other.

MATERIALS AND METHODS

Two species of prawns, *Palaemonetes vulgaris* and *P. pugio*, collected in the region of Woods Hole, Massachusetts, were used in this investigation.

The method used to determine the position of the distal retinal pigment was a slight modification of that devised by Sandeen and Brown (1952). The prawns were placed one at a time, ventral surface down, on the stage of the stereoscopic

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dissecting microscope. With the aid of an ocular micrometer and transmitted light the following measurements were made: (1) the width of the translucent portion of the compound eye in a plane parallel to the long axis of the eyestalk, and (2) the length of the eye from the corneal surface to the distal edge of the dorsal pigment spot at the base of the eye proper. Sandeen and Brown (1952) had measured to the proximal edge of this pigment spot. To render the distal clear portion of the eye more translucent and the proximal edge of this area more definite, the prawns were submerged in a dish of sea water on the stage of the microscope. The ratio of width of clear area (measurement 1) to total length (measurement 2) will be referred to as the distal retinal pigment index. Use of this ratio minimized the effect on the data of size differences. In a fully dark-adapted eye the distal retinal pigment abutted against the cornea, the mean distal retinal pigment index was 0.00. In a fully light-adapted eye the distal pigment index was about 0.25.

For all experiments wherein prawns were injected with tissue extracts the assay animals were placed into black enameled pans containing sea water about one inch deep. The pans were then placed at least one hour before the animals were injected under an illumination of such intensity that the distal retinal pigment was approximately midway between the fully light-adapted and dark-adapted positions. The light intensity used in each experiment will be stated below. Each extract was assayed on 10 prawns and the dose was 0.02 ml. per specimen. The assay animals had had one eyestalk removed at least 12 hours before use in an experiment. Because the eyestalk contained a large quantity of light-adapting hormone, one-eyed prawns would not be as readily able to antagonize injected dark-adapting hormone as would intact specimens.

Student's *t* test was used for determination of the level of significance between means. The 5% level was considered the maximum for a significant difference. Standard deviations and standard errors for the differences between means were also calculated. The results of the statistical analyses are summarized in Table I.

A Model E-800-2 Filter Paper Electrophoresis Apparatus manufactured by the Research Equipment Corporation, Oakland, California, was housed in a constant temperature room maintained at 43° F. on the days the electrophoretic analyses reported below were performed. For each experiment 40 eyestalks were extracted in 0.2 ml. distilled water. The extract was then centrifuged and applied with the aid of a hot air blower across a one-half-inch-wide Whatman No. 1 filter paper strip. The region of application was never more than ¼ inch long. This strip was then moistened with 0.1 *M* sodium hydroxide-boric acid buffer of pH 9.0 and placed into the migration chamber. After electrophoresis had proceeded for two hours at 500 V. and 0.1 mA. the strip was removed from the chamber. The region of application of the extract and one three-inch section from each side of the origin were placed into individual covered dishes containing 0.3 ml. sea water. These dishes were kept in the cold room for 30 minutes to allow materials to wash from the filter paper. As a control in the electrophoretic analyses whenever a strip was removed from the migration chamber a strip of filter paper three inches long was moistened with buffer and placed into 0.3 ml. sea water for 30 minutes in the cold room. The fluid from the containers was then collected in syringes for injection into assay animals.

TABLE I

Summary of the statistical analyses of the data presented herein. *N*, number of distal pigment indices used in the analysis; *S.D.*, standard deviation; *S.E.*, standard error of the difference between the means; *t*, Student's *t*; *p*, probability value. See text for complete explanation.

Analysis	N	Mean	Range	S.D.	S.E.	<i>t</i>	<i>p</i>
1 V	43	0.110	0.05–0.22	0.0356	0.00739	0.91	0.40
P	39	0.116	0.05–0.18	0.0326			
2 V	20	+0.034	(–0.02)–(+0.11)	0.0319	0.01044	0.73	0.50
P	20	+0.041	(–0.04)–(+0.11)	0.0340			
3 E	30	0.221	0.14–0.30	0.0468	0.0114	9.66	0.001
C	30	0.136	0.09–0.23	0.0395			
4 E	29	0.159	0.10–0.28	0.0363	0.00835	2.33	0.05
C	30	0.136	0.09–0.23	0.0268			
5 L	95	0.142	0.05–0.28	0.0376	0.00714	5.52	0.001
D	88	0.188	0.09–0.30	0.0519			
6 S	28	0.162	0.03–0.28	0.0754	0.0176	3.53	0.01
F	9	0.253	0.18–0.29	0.0309			
7 E	48	0.249	0.11–0.33	0.0578	0.01022	6.10	0.001
C	54	0.180	0.10–0.25	0.0432			
8 E	112	0.087	0.03–0.23	0.0311	0.00436	14.7	0.001
C	95	0.135	0.07–0.21	0.0299			
9 E	55	0.224	0.11–0.38	0.0474	0.00827	7.24	0.001
C	48	0.131	0.03–0.23	0.0362			
10 E	75	0.079	0.03–0.21	0.0311	0.00514	6.02	0.001
C	87	0.109	0.05–0.21	0.0342			
11 W	55	0.224	0.11–0.38	0.0474	0.00953	2.81	0.01
T	55	0.185	0.10–0.29	0.0524			
12 E	62	0.086	0.03–0.17	0.0359	0.00622	2.49	0.02
C	68	0.104	0.05–0.17	0.0348			

Analysis 1, Background adaptation of *P. vulgaris* (V) and *P. pugio* (P).

Analysis 2, Light-adapting potency of supraesophageal ganglia of *P. vulgaris* (V) and *P. pugio* (P).

Analysis 3, Supraesophageal ganglia of specimens two weeks in darkness (E) versus control (C).

Analysis 4, Supraesophageal ganglia of specimens two weeks in light (E) versus control (C).

Analysis 5, Supraesophageal ganglia of prawns in light (L) versus supraesophageal ganglia of prawns in darkness (D).

Analysis 6, Distal pigment index at start (S) and finish (F) of experiment.

Analysis 7, Eyestalk light-adapting hormone (E) versus control (C).

Analysis 8, Eyestalk dark-adapting hormone (E) versus control (C).

Analysis 9, Eyestalk light-adapting hormone (E) versus control (C).

Analysis 10, Eyestalk dark-adapting hormone (E) versus control (C).

Analysis 11, Comparison of light-adaptation produced by extracts of eyestalks without trypsin (W) and with trypsin (T).

Analysis 12, Dark-adaptation after electrophoresis of eyestalks (E) versus control (C).

EXPERIMENTS AND RESULTS

The distal retinal pigment of P. pugio and P. vulgaris

The specimens of *Palaemonetes* in the Woods Hole area had always been considered to be *P. vulgaris* (cf. Allee, 1923). However, Holthuis (1952) separated the prawns of the Woods Hole area into three species, *P. vulgaris*, *P. pugio* and *P. intermedius*. No investigator has reported specific differences of retinal pigment cells in *Palaemonetes*.

Examination of 451 specimens used in the present investigation revealed the presence of specimens of two of the species only, *P. vulgaris* and *P. pugio*. An overwhelming percentage (92.0%) was *P. vulgaris*. The present investigators used *Palaemonetes* without regard to species. This procedure was justifiable because the following two experiments revealed that the distal retinal pigments of *P. vulgaris* and *P. pugio* were physiologically indistinguishable.

(1) This experiment was designed to answer the following question. Are the mean distal retinal pigment indices of specimens of *P. vulgaris* and *P. pugio* exposed to the same intensity of illumination substantially the same? Forty-three specimens of *P. vulgaris* and 39 of *P. pugio* were placed into black enameled containers under an illumination of 31 ft. c. After one hour the distal retinal pigment index of each specimen was determined. The mean distal pigment indices were 0.110 for *P. vulgaris* and 0.116 for *P. pugio*. Statistical analysis of these data revealed no significant difference between the means (Table I, Analysis 1).

(2) The next experiment answered two questions. Do differences exist between the light-adapting potencies of extracts of supraesophageal ganglia from the two species? Are the responses of specimens of both species to the same extract different? Extracts with a concentration of 10 organs per ml. of sea water were prepared from supraesophageal ganglia of *P. vulgaris* and *P. pugio* and were assayed on 10 specimens of each species. The assay animals were in black enameled pans under an illumination of 31 ft. c. As a measure of the light-adapting potency of each extract, activity (potency) values were calculated in the following manner. The sum of the mean distal pigment indices, determined 30 and 60 minutes after the prawns were injected with the extracts, was subtracted from the sum of the means of the indices determined at the same time intervals with control specimens of the same species as the prawns injected with the extract of supraesophageal ganglia. The controls were injected with sea water. This difference between the sums was a measure of the potency of the extract. Thirty and 60 minutes were chosen because maximal light-adaptation in response to extracts of eyestalks and supraesophageal ganglia always occurred 30–60 minutes after injection. The activity values were as follows: "vulgaris" supraesophageal ganglia into "pugio," 0.068; "vulgaris" into "vulgaris," 0.068; "pugio" into "vulgaris," 0.076; "pugio" into "pugio," 0.082. An extract made from the supraesophageal ganglia of animals randomly selected from the stock aquaria and assayed on randomly selected assay animals was 0.071. The "vulgaris" into "pugio" and "pugio" into "pugio" potency values were analyzed statistically to determine if the difference between them was significant. These values were chosen because they were at the extremes. For the statistical analysis the mean distal pigment index of the control specimens was subtracted from each index obtained

from prawns injected with the extracts. The algebraic sign was noted. As anticipated, the two sets of data were not significantly different from each other (Table I, Analysis 2).

Influence of light and darkness upon the amount of light-adapting hormone in the supraesophageal ganglia

One group of prawns was placed in a darkroom, a similar group was placed into white enameled pans and exposed to a constant illumination of 31 ft. c. After 14 days the supraesophageal ganglia of specimens of both groups were ex-

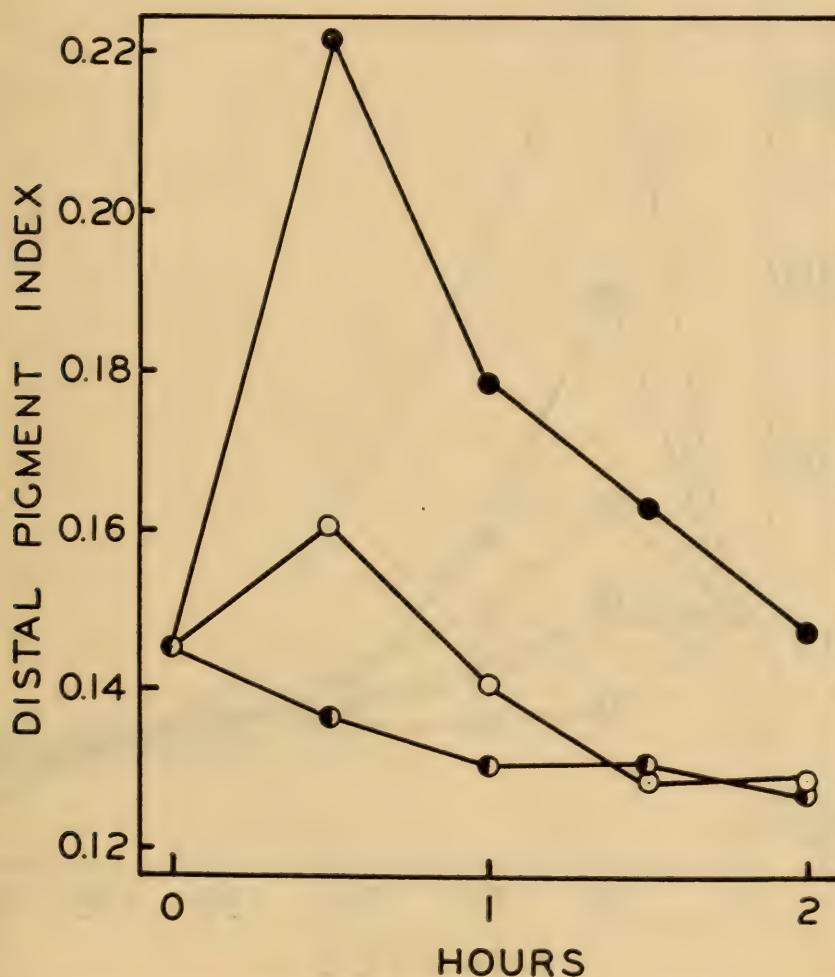


FIGURE 1. Responses of the distal retinal pigment to extracts of supraesophageal ganglia of prawns maintained on a white background under an illumination of 31 ft. c. (circles) and in darkness (dots) for 14 days. Control, half-filled circles.

tracted in sea water (10 organs/ml.) and assayed on one-eyed prawns in black enameled pans under an illumination of 31 ft. c. Control specimens received injections of sea water.

Extracts of the supraesophageal ganglia of both groups produced a light-adaptational response. However, the responses of the assay animals to the organs of specimens that had been in darkness was much greater than that produced by the extracts of supraesophageal ganglia of specimens that had been

illuminated. The experiment was done two more times. The results were qualitatively the same as those obtained the first time the experiment was done. Data of the three experiments were consequently averaged and the means were used in the preparation of Figure 1. The light-adaptational responses of both groups of assay prawns were statistically significantly different from the controls

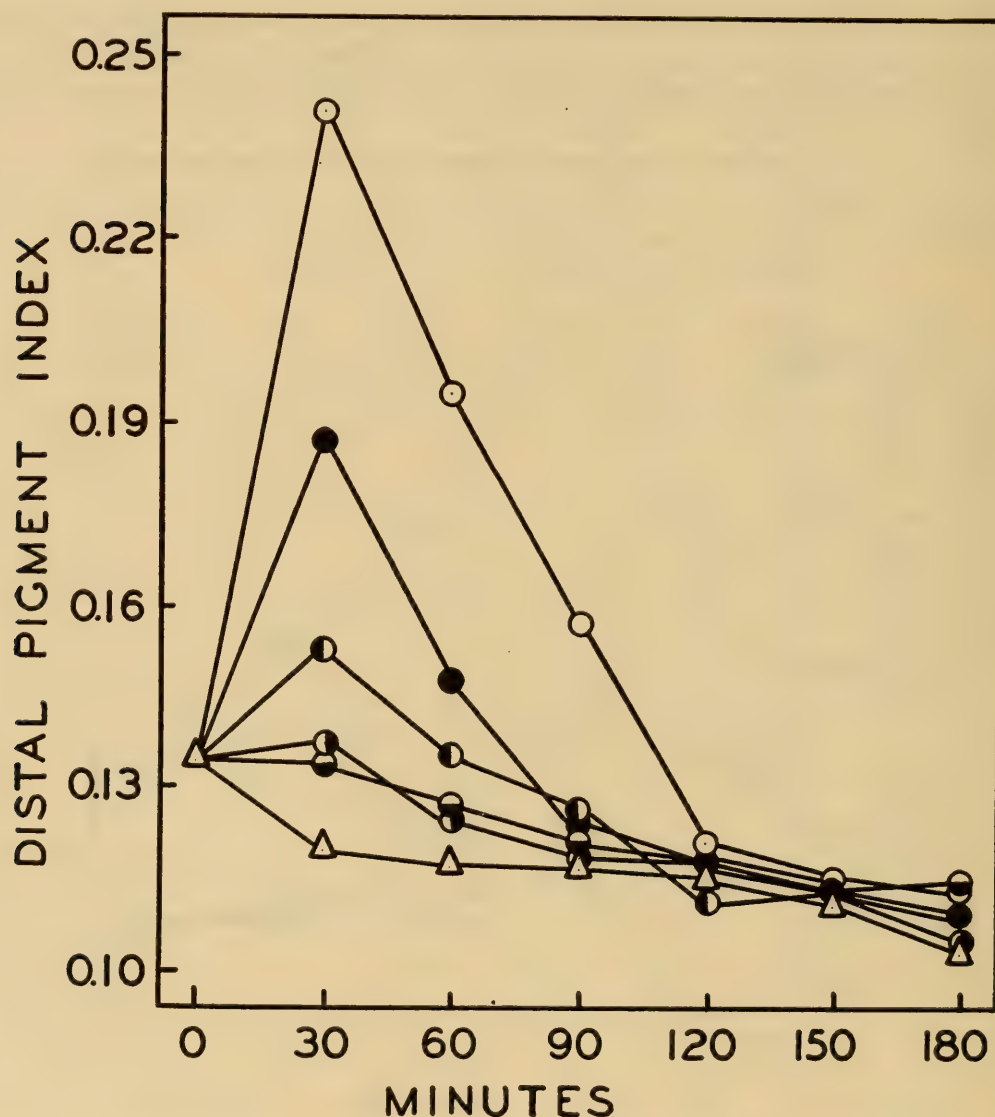


FIGURE 2. Response of the distal retinal pigment to several concentrations of extracts of supraesophageal ganglia of animals collected less than 24 hours before use. Circles, one organ per 0.02 ml. sea water; dots, $\frac{1}{2}$ organ per 0.02 ml.; circles half-filled on left, $\frac{1}{4}$ organ per 0.02 ml.; circles half-filled on right, $\frac{1}{8}$ organ per 0.02 ml.; circles half-filled on bottom, $\frac{1}{16}$ organ per 0.02 ml.; triangles, control of sea water.

(Table I, Analyses 3, 4). The distal pigment indices determined 30 minutes after the extracts had been administered were used in the statistical analyses. The difference between the responses of assay animals to the extracts of the supraesophageal ganglia of the specimens that had been in light and in darkness was also highly significant (Table I, Analysis 5). Analysis 5 was based on the

distal pigment indices determined 30, 60 and 90 minutes after the extracts had been injected.

The next experiment was designed to learn how the amount of light-adapting hormone in the supraesophageal ganglia of freshly collected specimens compared with the amounts of this hormone in the supraesophageal ganglia of prawns that had been in darkness or in light for 14 days. Supraesophageal ganglia were removed from prawns that had been collected less than 24 hours prior to use in the experiment and triturated with sufficient sea water to yield extracts that contained $\frac{1}{16}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$ and 1 organ per 0.02 ml. sea water. Each extract was assayed on 10 one-eyed prawns in black pans under an illumination of 31 ft. c. The responses were observed for three hours. This experiment was performed three times. The data of the three experiments were then averaged and the means were used in the preparation of Figure 2.

Two facts were apparent from inspection of the figure: (1) the higher the concentration of supraesophageal ganglia in the extracts, the greater the distal pigment index 30 minutes after the extracts were injected, and (2) the rate of return of the index to the control level was faster with the higher concentrations than with the lower ones. Furthermore, no indication of a dark-adapting response was apparent even at the highest concentration.

As a measure of the light-adapting potency of the extracts the differences between the means of the distal pigment indices of the controls and of prawns receiving extracts of supraesophageal ganglia were summed. The sums (potency values) were used to compile Figure 3. In like manner, potency values were calculated for the extracts of the supraesophageal ganglia of the specimens that had been in light (0.034) and in darkness (0.204) for 14 days. The coordinates for the supraesophageal ganglia of specimens that had been in light 14 days (0.2 organ/0.02 ml. and a potency value of 0.034) almost fell on the curve of Figure 3. As the curve was drawn, the potency value expected from an extract containing two-tenths of the supraesophageal ganglia of one prawn per 0.02 ml. was 0.045. Illumination, therefore, had no appreciable effect on the amount of light-adapting hormone. Further inspection of the figure revealed that a potency value of 0.204 would be caused by an extract of supraesophageal ganglia of freshly collected prawns with a concentration of eight-tenths of an organ per 0.02 ml. Presumably, therefore, the amount of light-adapting hormone in the supraesophageal ganglia quadrupled during the two weeks the specimens had been in the dark-room.

Effect of time in darkness on the ability of the distal retinal pigment to respond to a dark-to-light change

The object of this experiment was to determine if the period of time specimens were kept in darkness influenced their subsequent rate of light-adaptation. Prawns were collected, put into white enameled pans and placed in darkness. Two hours later the prawns were exposed to an illumination of 31 ft. c. for 30 minutes. The distal pigment indices of 10 specimens were determined and the prawns were again put in darkness. They were subsequently exposed to illumination after having been in darkness 3, 5, 7, 10, 12 and 14 days. The mean distal pigment indices determined after 30 minutes of illumination gradually increased.

The experiment was repeated and the results were substantially the same. The data of the two experiments were averaged and the means were used in the preparation of Figure 4. The number of prawns gradually decreased while each experiment was in progress. Totals for both experiments were: 20 on the first



FIGURE 3. Relationship between light-adapting potency and concentration of extracts of supraesophageal ganglia of specimens collected less than 24 hours before use. The concentration is the logarithm of the number of supraesophageal ganglia each assay animal received triturated in 0.02 ml. sea water.

and third days, 16 on the fifth day, 15 on seventh and tenth days, 12 on the twelfth day and nine on the last day of the experiment.

The indices recorded on the first and last days of both experiments were analyzed statistically (Table I, Analysis 6); the difference between the means was highly significant. The longer the prawns had been in darkness, the faster the distal pigment approached the fully light-adapted position.

Digestion of the light-adapting and dark-adapting hormones by trypsin

The object of the first experiment of this series was to demonstrate that a boiled solution of trypsin would not inactivate the retinal pigment hormones. After boiling, the enzyme would have been inactivated but some contaminant might have possibly destroyed the hormones. This possibility had to be ruled out. For the first experiment of this group a 2% solution of trypsin in sea water was prepared; at the same time a freshly prepared extract of eight eyestalks in 0.2 ml. sea water was boiled for 30 seconds and centrifuged. Equal volumes of the boiled trypsin solution and the eyestalk extract were mixed together. The

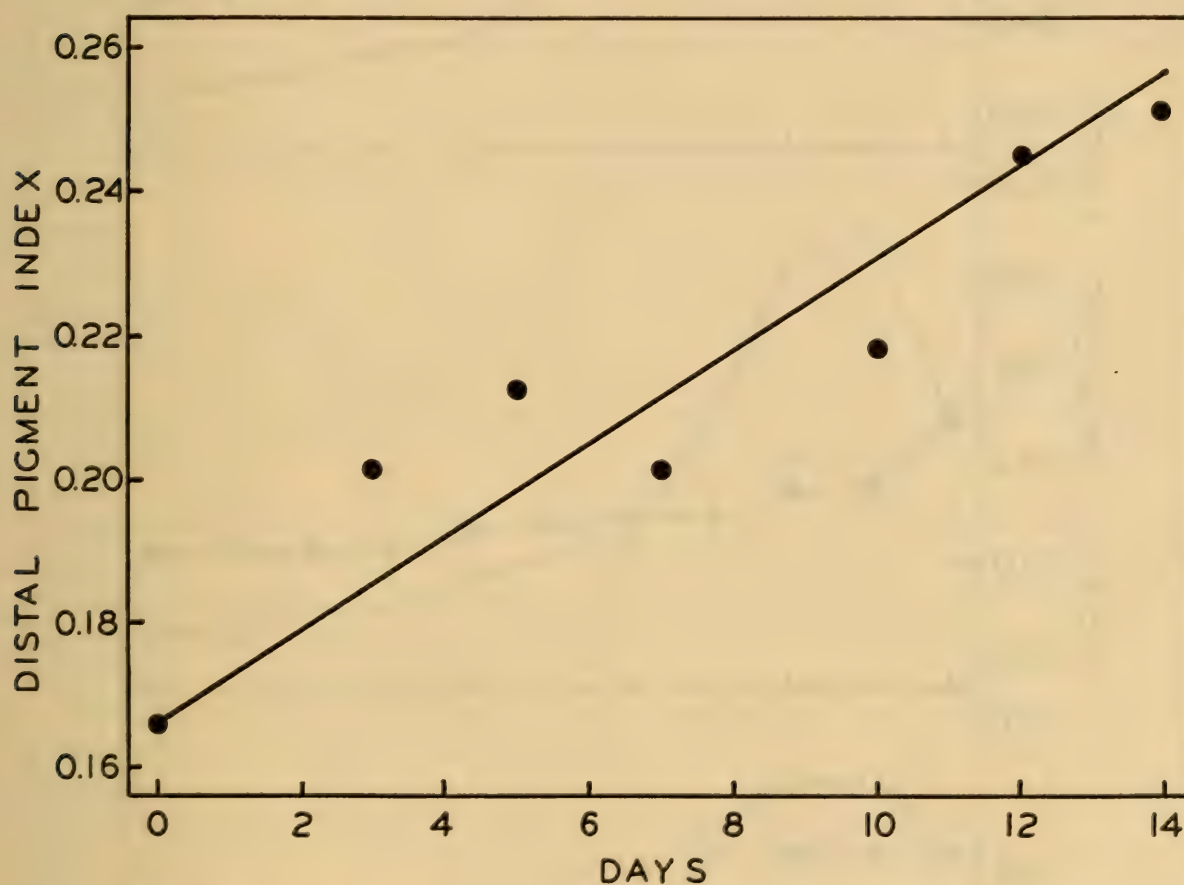


FIGURE 4. The distal retinal pigment index 30 minutes after prawns had been taken from darkness and placed on a white background under an illumination of 31 ft. c., versus the number of days the specimens had been in darkness.

mixture was then injected into one-eyed prawns in black pans under an illumination of 22 ft. c. Boiled trypsin solution diluted with an equal volume of sea water was injected into the controls.

The results were those that were anticipated. A light-adaptational response occurred and lasted two hours. A dark-adaptational response followed. The extent of the latter was not followed to its completion. Fingerman, Lowe and Sundararaj (1959) reported that the dark-adaptational response lasted at least five hours. The experiment was performed two more times and the results were

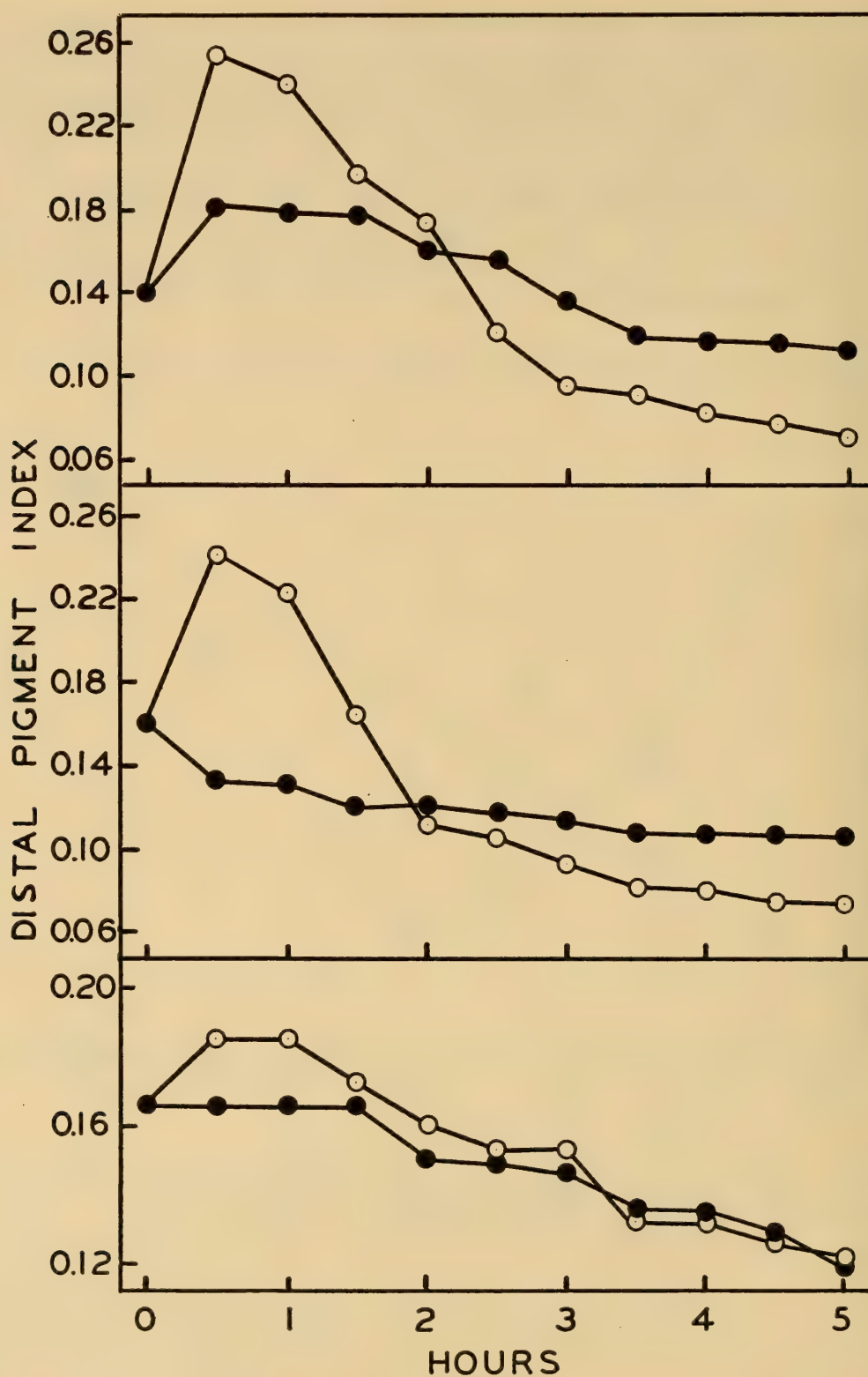


FIGURE 5. Responses of the distal retinal pigment to extracts of eyestalks. Top, the extract was prepared in boiled, centrifuged trypsin solution, circles. Control of boiled, centrifuged trypsin in sea water, dots. Middle, extract prepared in sea water and kept for one hour, circles. Sea water control, dots. Bottom, extract in trypsin solution for one hour, circles. Control, dots.

the same. The data of the three experiments were averaged and the means were used in the preparation of the top portion of Figure 5.

These data were analyzed statistically (Table I, Analyses 7, 8) and the amounts of light-adaptation and dark-adaptation were significant. The distal pigment indices obtained 30 and 60 minutes after the extracts were injected were used for Analysis 7; the indices determined 3.0, 3.5, 4.0 and 4.5 hours after the extracts were injected were used in Analysis 8.

For the next experiment an extract was prepared of 18 eyestalks in 0.45 ml. sea water. Four-tenths ml. of the centrifuged extract was divided into two equal portions. To one fraction was added 0.2 ml. sea water and to the other 0.2 ml. of 2% trypsin solution in sea water. Both tubes were kept for one hour at room temperature, 24° C., at which time the material in the tubes was boiled for 30 seconds and centrifuged. The supernatant from both tubes was injected into assay animals. Boiled, centrifuged trypsin solution was injected into one-eyed prawns as a control for the extract of eyestalks treated with trypsin. As a control for the extract not treated with trypsin, sea water was injected into one-eyed prawns.

The extract that did not contain trypsin caused light-adaptation and subsequent dark adaptation. The trypsin-treated extract caused a small amount of light-adaptation and no dark-adaptation. The experiment was performed two more times and each time the results were essentially the same. The data of the three experiments were averaged and the means were used in the preparation of the middle (pure extract) and bottom (trypsin-treated extract) portions of Figure 5.

Statistical analyses (Table I, Analyses 9, 10) of the results depicted in the middle portion of Figure 5 showed that the light-adapting and dark-adapting effects were significant. The distal pigment indices determined 30 and 60 minutes after the extracts were administered were used for Analysis 9, the indices obtained after 4.0, 4.5 and 5.0 hours for Analysis 10.

To determine if the difference between the degrees of light-adaptation produced by the pure and trypsin-treated extracts was significant, the distal pigment indices determined 30 and 60 minutes after injection of the extracts were analyzed statistically (Table I, Analysis 11) and found to be significant at the 1% level. The amount of light-adapting hormone in the extract had been reduced by the trypsin.

Electrophoretic analysis of the retinal pigment hormones

The object of the last set of experiments was to separate the dark-adapting hormone in the eyestalk from the light-adapting one. Separation was accomplished by filter paper electrophoresis performed in the manner described above. The experiment was performed three times.

The results of the three experiments were qualitatively alike and were averaged (Fig. 6). The region of application of the extract to the strip and the three-inch section of the strip on the cathodal side of the origin contained light-adapting and dark-adapting substances. Fluid obtained from the three-inch section on the anodal side of the origin produced dark-adaptation alone. Distal pigment indices determined 90, 120 and 150 minutes after the extracts from the anodal portion

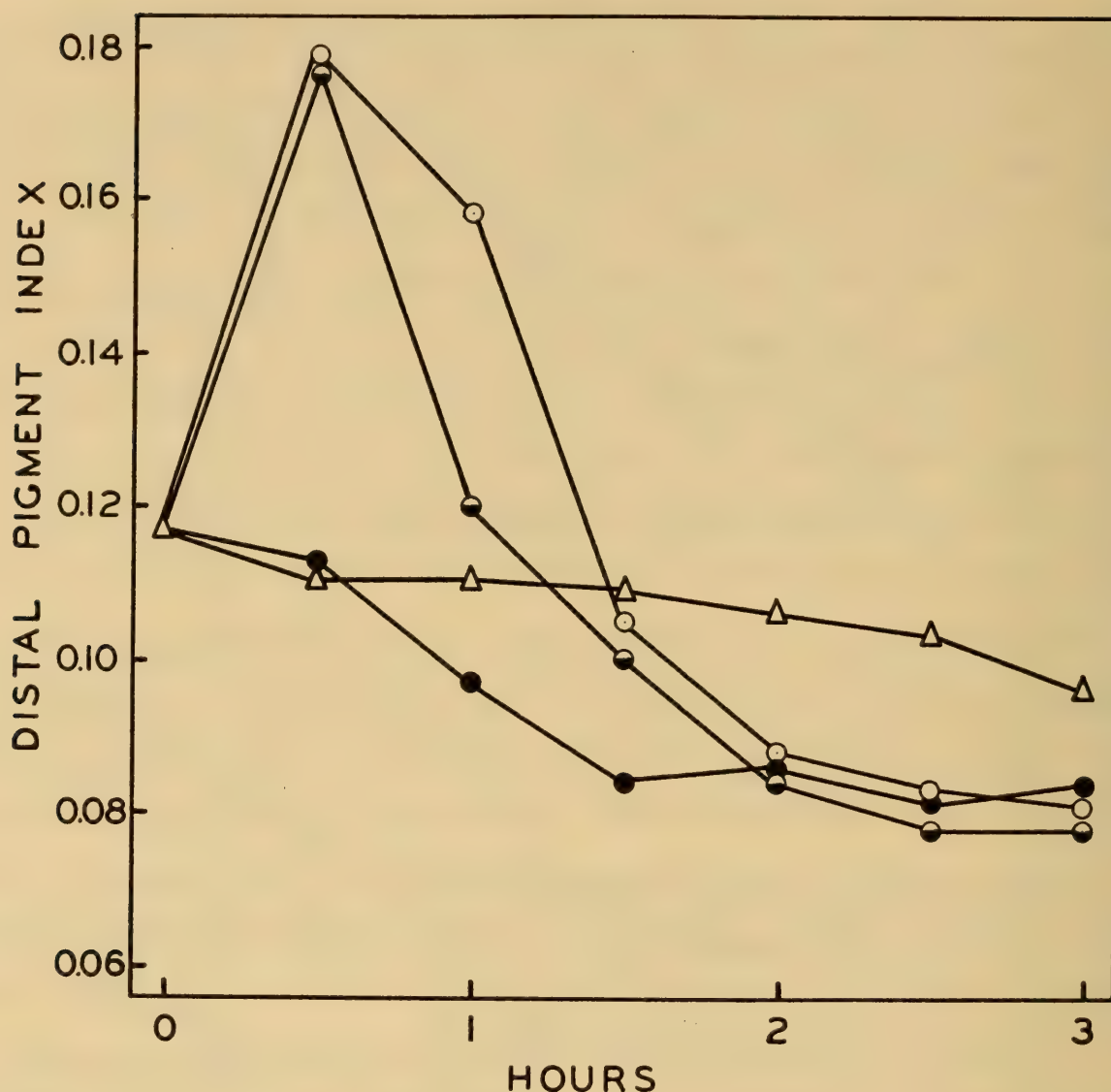


FIGURE 6. Responses of the distal retinal pigment to extracts of eyestalks. These extracts had been subjected to filter paper electrophoresis. Circles, fraction that migrated toward the cathode; dots, fraction that migrated toward the anode; half-filled circles, fraction that remained at the origin; triangles, control.

of the strips and the control strips had been injected were compared statistically (Table I, Analysis 12). The amount of dark-adaptation was highly significant.

DISCUSSION

The amount of light-adapting hormone in the supraesophageal ganglia increased in specimens of *Palaemonetes* kept in darkness. This hormone in the supraesophageal ganglia, therefore, must be normally involved in regulation of the distal retinal pigment. The site of release of the light-adapting hormone from the supraesophageal ganglia is unknown. It could be transported via axons to a storage and release center in the eyestalks (*e.g.* sinus gland) or could be released directly from neurosecretory cells in the supraesophageal ganglia into the blood.

The light-adaptational response produced by the strongest extract of supraesophageal ganglia (Fig. 2) was of the same magnitude as that produced by the extracts of eyestalks (Fig. 5). However, the extracts of the supraesophageal ganglia did not cause a dark-adaptational response. The dark-adaptational response produced by the eyestalk extracts might have been considered overcompensation by the prawns in removing the injected light-adapting hormone from the blood. This interpretation, however, can not be correct in view of the results obtained with the extracts of the supraesophageal ganglia (Fig. 2).

The data shown in Figure 4 reveal that the prawns adapted faster to light the longer they had been in darkness. The observation that the supraesophageal ganglia of prawns kept in a darkroom for 14 days contained more light-adapting hormone than the organs of illuminated specimens can explain the results shown in Figure 4. The animals light-adapted at a faster rate with increased time in darkness because they had a greater store of light-adapting hormone to use.

Some physico-chemical properties of the retinal pigment hormones of *Palaemonetes* can be adduced from these experiments. Both hormones are heat-stable. With regard to the chemical nature of the retinal pigment hormones, there is inconclusive evidence that the neurosecretory products of crustaceans are polypeptides. The sensitivity of these substances to trypsin and chymotrypsin suggests the presence of peptide bonds. Pérez-González (1957) found that the hormone that dispersed the black pigment in the fiddler crab, *Uca pugilator*, was inactivated by chymotrypsin. Kleinholz (personal communication) found that the light-adapting substance in the eyestalk of *Palaemon serratus* at Naples, Italy, was sensitive to trypsin and chymotrypsin. Evidence was presented in Figure 5 for the trypsin sensitivity of the retinal pigment dark-adapting and light-adapting hormones in *Palaemonetes*. Trypsin and chymotrypsin, however, also have weak esterase activity and inactivation by these enzymes, alone, is not sufficient proof of the peptide nature of these neurosecretory substances.

Both retinal pigment hormones migrated in an electric field (Fig. 6). At pH 9.0 the dark-adapting hormone separated from the light-adapting hormone so that light-adaptation did not precede the dark-adaptational response. In contrast, whenever extracts of eyestalks were injected before electrophoresis a light-adaptational response occurred before the dark-adaptational one (top portion of Figure 5). Because the dark-adapting hormone was found at the origin and on both sides of it, pH 9.0 must be close to its isoelectric point. The isoelectric point of the light-adapting hormone must be higher than pH 9.0 because no light-adapting hormone was found on the anodal side of the origin.

SUMMARY AND CONCLUSIONS

1. Some physico-chemical properties of the hormones activating the distal retinal pigment of the prawn *Palaemonetes* were determined.

2. The light-adapting and dark-adapting hormones were heat-stable, inactivated by trypsin and migrated in an electric field. These findings suggest that both hormones are polypeptides.

3. The amount of light-adapting hormone increased four-fold in the supraesophageal ganglia of prawns kept in darkness 14 days. The quantity of this hormone in the supraesophageal ganglia of specimens illuminated for 14 days did

not change detectably. These results demonstrated that the light-adapting hormone from the supraesophageal ganglia was used by the prawns in regulating the distal retinal pigment.

4. The longer prawns were kept in darkness, the faster was the rate of migration of the distal pigment toward the light-adapted position when prawns were illuminated. This increase was explained by the increase in the light-adapting hormone in the supraesophageal ganglia of specimens kept in darkness.

5. The dark-adapting hormone was separated from the light-adapting one by filter paper electrophoresis at pH 9.0.

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SEX-LINKED INHERITANCE IN ULVA

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In an earlier report (Föyn, 1959) mention was made of two autosomal mutants, Slender (*S1*) and long (*l*), both of which spontaneously arose in cultures of *Ulva mutabilis* and both of which exhibit sex-controlled manifestation. Found among the young gametophytes from a crossing between the two mutants was a small dwarfed individual entirely different from the types hitherto known. The individual was isolated and proved to belong to the — -sex. In order to determine whether it was a matter of a new hereditary type or only a phenotypical abnormality, the gametes were put to parthenogenesis. The new germ plants were all of the same kind as the mother plant, and thus probably represented a new mutant. As described earlier (Föyn, 1958) parthenogenesis in this species is normally connected with doubling of the chromosome number. The parthenogenetic plants therefore became diploid and produced (haploid) zoöspores. Figure 1 shows a few stages in their development towards gametophytes.

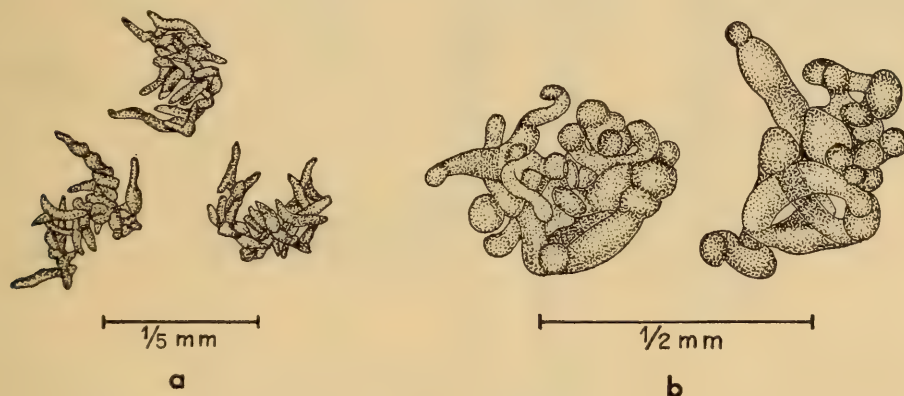


FIGURE 1. *Ulva mutabilis*. Young *dw S1* individuals from zoöspores.
a, 10 days old; b, 17 days old.

The gametophytes were fertile after a good month and they were then crossed with the wild-type. The zygotes germinated immediately to germ plants of the sporophyte generation. As Figure 2 shows, they resemble the sporophyte germ plants after crossing between the Slender-type and the wild-type but diverge from them by the tapered top and the weakly developed rhizoids which do not grow out from the entire basal part, but only from a single point medially below this. With some germ plants the basal part can also grow one or a few short branches such as in the individual on the right of the figure, but this is rare.

The hybrid sporophytes when about 2½ months old formed zoöspores. The plants had then grown to a length of 25–30 cm., flat, and with even width (ca. 1 cm.) band. Zoöspores from one of the plants were transferred to a petri dish

which was thereupon placed in darkness until the following day. The zoöspores had by then become distributed evenly over the bottom of the dish and had affixed themselves to the glass. Inspection 10 days later found the bottom over-grown by various types of germ plants. A precise count was taken within a circle-shaped area of 2.3 cm. diameter, and the various types were at the same time separated, each plant being loosened from the under-bedding with the aid of a glass needle and transferred to a new petri dish. Altogether, within the limited area 728 germ plants were found. Of these, 189 belonged to the wild-



FIGURE 2. *Ulva mutabilis*. Twelve-day-old sporophytes from a cross between a *dw S1* gametophyte and a gametophyte of the wild-type.

type and 138 to the Slender-type. The remaining 401 consisted for the greater part of germ plants of the same appearance as in Figure 1a and thus resembled the pristine mutant plant completely. A smaller number were more simple, built more clumpily, and were only weakly ramified (Fig. 3a). Every transition between the two types was found, however, and an assortment into two distinct groups had, therefore, to be abandoned. All of them were reckoned as belonging to the mutant type. Thirty-six germ plants could not be identified; they were, partly, of different shape and dissimilar to the types hitherto known. The plants

were accordingly carried forward to sex-maturity and the gametes were put to parthenogenetic development. A survey of the parthenogamete plants 14 days later showed that not one of them represented new mutants. Four belonged to the wild-type, 21 to the Slender-type and 11 to the mutant-type. The final result of the count was therefore:

wild-type:	193 individuals (26.51%)
mutant-type:	376 individuals (51.65%)
Slender-type:	159 individuals (21.84%)

It is therefore a matter of a crossing with two independently segregating factors of which the one is *S1*. The other has been designated *dw*. Half of the 376 germ plants of the mutant type must have both the chromosomes with *S1* and the chromosome with *dw* the other half, only the chromosome with *dw*.

Three dishes with altogether 220 mutant germ plants were placed under the source of light and, gradually, as the plants grew, the largest were isolated, carried forward to sex-maturity, and crossed with the wild-type. It soon became

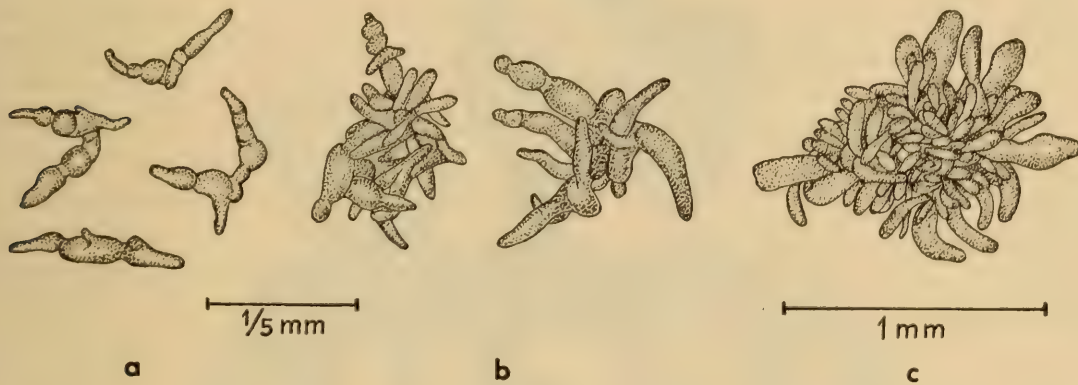


FIGURE 3. *Ulva mutabilis*. Young *dw* individuals from zoöspores. a, 12 days old; b, 19 days old; c, 30 days old.

evident that *dw S1* individuals grow much more rapidly and, therefore, become fertile earlier than *dw*-individuals. The first crossings all resulted in sporophytes of the same kind as the mother sporophyte (Fig. 2). It was only after 84 plants had been tested that the crossings between the three successive individuals and the wild-type gave another result, namely, sporophytes of the wild-type. Upon the formation of zoöspores eleven weeks later, they segregated into the mutant and the wild-type in the ratio 1:1. A precise count was not taken as already at the first glance into the microscope it was evident that the two types were represented equally strongly.

For each crossing undertaken, the gametes of the mutant partner were put to parthenogenesis. In the first 84 cases mentioned the gametes developed, as expected, mainly to germ plants of the ramified type, but also a few developed to individuals of the simple type. After the three crossings following, however, the ratio was reversed: most of the parthenogamete plants became simple whereas only a few became considerably ramified. Even if weak or no ramification accordingly must be said to be a typical character of the pure *dw* germ plant,

it is no certain distinguishing feature. One of the three above-mentioned dishes having mutant germ plants contained only individuals of the simple type, altogether 20; 13 of them later yielded gametes, 10 proved to be pure *dw* plants and the remaining three, *dw S1* individuals. The two other dishes each contained 100 germ plants, chosen casually, without regard to type or size. Of these, altogether 197 have been tested in crossing with the wild-type with the following result: 118 were *dw S1* individuals, 79, *dw* individuals.

It is apparent from Figures 3c and 4 that the *dw* plants also later become considerably ramified, frequently much more so than *dw S1* plants. The fertile *dw* individual looks like a rosette of young wild-type plants grown together. The thallus is dark green, often folded, and gives a stunted impression. The two layers of cells are either grown together throughout or are separated from each other along the border of the thallus. The thickness of the layers is greater than

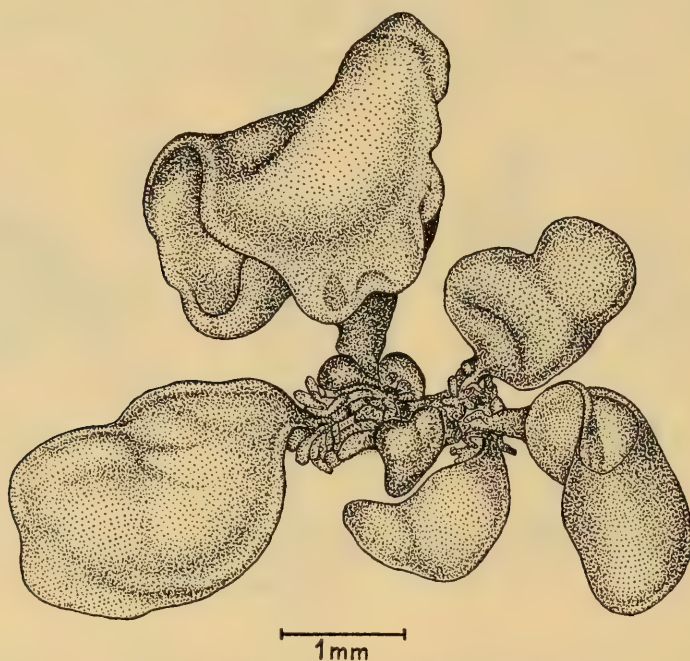


FIGURE 4. *Ulva mutabilis*. A full grown and fertile *dw* sporophyte. Three months old.

in the wild-type. The rhizoids are weakly developed or are not at all distinguishable. It is because of this dwarf-like appearance that the mutant factor has been termed *dw*.

The fertile *dw S1* plants much resemble the *dw* plants. Nor do they become larger than these but the two layers of cells are often free throughout and, partially, distend strongly from each other. Most frequently, they have the usual green colour.

More interesting than these results, however, was that all the 210 mutant individuals (both *dw* and *dw S1* individuals) which had been carried forward to sex maturity proved to belong to the —-sex. Investigation was therefore made as to how the 193 individuals of the wild-type and the 159 Slender individuals behaved in this respect. Where the latter are concerned, it had already been

observed during the counting that most of them were ramified which, with this mutant, is a quite certain sign that they are + -individuals (see below). Only 61 were unramified and these were therefore isolated and carried forward to sex maturity. All of them revealed themselves as + -plants. Of the wild-type, 100 individuals were tested with regard to sex, all with the same result, namely, that they belonged to the + -sex. Also in different later crossings which, in another connection, have been performed with *dw*-plants, individuals with the *dw* factor have proved to be of the - -sex. The factor *dw* must therefore be located in the same chromosome as the factor(s) for the - -sex.

It has not been possible to establish cases of crossing-over between *dw* and the sex-factor(s). The crossing best suitable for the discovery of such cases is that between *dw S1* and *dw⁺ S1* individuals. The crossing can naturally only result in the same types of gametophytes as the parents, and, apart from eventual cross-overs, the *dw S1* plants will belong to the - -sex and the *dw⁺ S1* plants, the + -sex. As described earlier (Föyn, 1959) the germ plants of the - -sex of the Slender race are always long, slim and unramified, whereas the germ plants of the opposite sex can either be like the - -individuals or be small and considerably ramified. This variation rests upon hitherto unknown environmental conditions, as + -zoöspores from one and the same sporophyte after one swarming can develop to germ plants, all of which are small and ramified, and, after another, to both ramified and unramified, or to unramified germ plants only. In the cases where the first possibility is realized from the + -zoöspores of *dw dw⁺ S1 S1*-sporophyte, naturally a - -individual of the Slender-type will immediately become evident. Of the 14 petri dishes with their bottom wholly covered by haploid germ plants from two *dw dw⁺ S1 S1*-sporophytes, three contained exclusively small and ramified germ plants. In the one dish the germ plants originated from the one, and, in the other two, from the second sporophyte. In two of the dishes there were more than 4000 individuals, in the third, more than 5000 individuals; thus, from among more than 6500 *dw⁺ S1*-individuals, not a single cross-over was observed.

The author would like to express his thanks to Miss Ingeborg Gjören for the execution of the drawings, and to Mr. John C. Aird for the translation of the manuscript.

SUMMARY

A recessive sex-linked mutant is described that arose spontaneously in cultures of *Ulva mutabilis*. It has not been possible to establish cases of crossing-over between the sex chromosomes.

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INFLUENCE OF CARBONIC ANHYDRASE INHIBITORS ON SHELL GROWTH OF A FRESH-WATER SNAIL, *PHYSA HETEROSTROPHA*¹

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The enzyme carbonic anhydrase catalyses reversibly the reaction



(Berliner and Orloff, 1956) and has been shown to accelerate both deposition and dissolution of calcium carbonate *in vitro* (Meldrum and Roughton, 1933). In view of its possible implication in shell formation, the mantle tissues of molluscs have been examined and the enzyme demonstrated in several of them (Florkin and de Marchin, 1941; Maetz, 1946; Freeman and Wilbur, 1948; Wilbur and Anderson, 1950; Stolkowski, 1951; Tsujii and Machida, 1953; Ikinago, 1954; Kawai, 1954a, 1954b; Larraneta and Ponz, 1954; Clark, 1957). However, among 20 species examined, Freeman and Wilbur (1948) found little or no carbonic anhydrase activity in mantle tissue in two, a pelecypod and a gastropod, both shell-formers, and Stolkowski (1951) found none in the mantles of *Ostrea edulis* and several other shell-forming molluscs. Such results suggest that at least in some species the enzyme is not essential for shell formation.

Stolkowski (1951) apparently first used a carbonic anhydrase inhibitor, benzenesulfonamide, in studies of the role of the enzyme in molluscs, observing decreased shell regeneration in *Helix aspersa* in the presence of the drug. He concluded that the enzyme is important in determining the crystalline form in which calcium carbonate appears in the shell. Wilbur and Jodrey (1955) found that two carbonic anhydrase inhibitors, 2-benzothiazolesulfonamide and Diamox (2-acetyl-amino-1,3,4-thiadiazole-5-sulfonamide), markedly decrease the rate of deposit of Ca^{45} in the shell of *Crassostrea virginica*. Abolins-Krogis (1958) in a study of shell regeneration in *Helix pomatia* concludes on the basis of as yet unpublished data on carbonic anhydrase inhibition that (p. 36) the enzyme "is necessary for the formation of calcium carbonate crystals in the regenerating shell." These studies suggest a role of carbonic anhydrase in shell formation but are inconclusive in establishing it. The two inhibitors used by Wilbur and Jodrey, 2-benzothiazolesulfonamide and Diamox, exhibited quite different extents of inhibition of Ca^{45} deposition in the intact animal, only Diamox affecting deposition in a manner consistent with the hypothesis that the results were due to carbonic anhydrase inhibition. Even though in the mantle-shell preparation used in part of the studies the two drugs had effects consistent with the idea that the enzyme is important in determining the rate of calcium deposit, the side effects of one of the drugs suggest caution in interpretation of the data. Further, the

¹ This study has been supported in part by a grant from the Southern Fellowships Fund.

data are consistent with another interpretation, that carbonic anhydrase influences the exchange of calcium between the shell and the extrapallial fluid. This is an exchange which may not include the reactions which serve as a pathway for shell formation.

The study of Abolins-Krogis (1958) indicates that calcium carbonate crystals did not develop in the presence of a carbonic anhydrase inhibitor. Since the reaction catalyzed by the enzyme occurs at a very appreciable rate, proceeding 90 per cent to equilibrium in 200 seconds (Berliner and Orloff, 1956), it would be expected that during inhibition of the enzyme deposition of calcium carbonate would occur at a decreased rate but would not cease.

One method of resolving the dilemma would be to study the growth of shell in the presence of a number of carbonic anhydrase inhibitors, for if a series of side effects were operative among the drugs in decreasing shell development these effects should show different extents or at least statistically modify in different ways the pattern for the growth of the populations treated with different drugs. On the other hand, if the effect on shell development is statistically similar in the case of several known carbonic anhydrase inhibitors this would strengthen the evidence for a direct role of the enzyme in shell development. In the present study the hypothesis that carbonic anhydrase is important in molluscan shell development has been tested by a study of the influence of five carbonic anhydrase inhibitors on the growth of the shell of the pond snail, *Physa heterostropha*.²

METHODS

Adult snails of a local population were maintained as stock cultures on lettuce with oyster shell chips in the bottom of aquaria. Young snails not over a month old were removed from the stock aquaria as required and placed in 4-inch fingerbowls containing 100 ml. water which had been equilibrated with precipitated calcium carbonate for at least a week. Three or four snails from about 2 to 5 mm. in length, which were individually distinguished in the course of measurements, were placed in each fingerbowl along with lettuce. Twenty-four hours later each snail was measured, using a micrometer calibrated to 0.01 mm., and the water was replaced. At this time appropriate volumes of solutions of the drugs (made up in the CaCO₃-saturated water) were substituted for a part of the water in dishes containing the experimental animals. Subsequently the water or inhibitor solution was replaced daily and lettuce was added as needed. (For information on feeding, see Table I.) The snails were measured on alternate days for ten days. In the few cases in which snails were lost, died or were accidentally killed, the measurements were included if data were available for six or more days of growth. (Such losses were: 8 snails died during the major series of observations, 6 were killed accidentally during handling, and there were 3 losses which were unaccounted for among 672 snails.)

The experiment was carried out in the room in which the snails were reared, at a temperature which in most experiments approximated 26° C. (In a preliminary study the temperature approximated 21°, as indicated in appropriate places in the tables.)

² The author appreciates the identification of the snail by Dr. H. Van der Schalie of the University of Michigan.

TABLE I

Growth of Physa heterostropha fed in three ways. Data for snails having median lengths during growth periods of 3.00–5.99 mm.

Feeding	No. snails	Growth (mm./day)		
		Median	Mean	Range
I	12	0.11	0.10 $\pm$ 0.015	0.05–0.13
II	55	0.25	0.27 $\pm$ 0.010	0.07–0.38
III	30	0.40	0.41 $\pm$ 0.014	0.28–0.49

Observations were at *ca.* 26°. Fresh lettuce was available to all snails continuously, the amount of decaying lettuce varying. In feeding method I, each morning any piece of lettuce showing signs of decay was removed and replaced with fresh lettuce. In method II, each piece of lettuce was left until all parts showed signs of decay. Then it was removed and replaced with fresh lettuce. In method III, decaying lettuce was available at all times and decaying pieces were removed when only plant fibers remained.

The inhibitors used were Diamox (2A),³ 2-benzothiazolesulfanilamide (2B)³ (Miller, Dessert and Roblin, 1950), benzenesulfonamide (BS), p-toluenesulfonamide (TS) (Krebs, 1948) and sulfanilamide (SA) (Mann and Keilin, 1940).

RESULTS

Examination of preliminary data in graphs indicated approximately uniform growth rates for snails having median lengths ranging from 2 to 6 mm. during growth periods and lower rates for those having lengths outside this range. More critical examination of the data and subsequently of data for all control groups, using the Kruskal-Wallis test (Kruskal and Wallis, 1952), indicates the range of lengths having statistically uniform growth rates is more restricted, from 3.00 to 5.99 mm. when data are examined in classes having 0.50 mm. ranges. All data for controls have been examined and, with the exception of a single class among those subjected to feeding method I, show no statistically significant variations in growth rates among classes having median lengths of 3.00 to 5.99 mm. within any method of feeding at a given temperature. As determined by the Kruskal-Wallis test, probability (*p*) for controls ranges from 0.3 to 0.8, with the exception indicated. Statistically significant variations among classes within experiments were obtained in each case in which a broader range of median lengths was used. Inclusion of all data in calculations would have made no difference in the over-all picture, for among the control experiments the mean growth rates of snails subjected to different feeding regimes would have been 0.10, 0.24 and 0.37 instead of 0.10, 0.25 and 0.41, and similar differences were found for experimental populations. The difference is in part determined by the proportion of measurement made on snails outside the range 3.00 to 5.99 mm.

Preliminary experiments indicated that the manner of feeding greatly influenced the rate of growth. Although fresh lettuce supported slow growth, the most rapid growth was obtained only if decaying lettuce was available to the

³ Diamox and 2-benzothiazolesulfanilamide were supplied by Dr. Emmanuel Waletzky of American Cyanamid Co.

TABLE II

Growth of Physa heterostropha in 2-benzothiazolesulfonamide. (Data for periods in which median lengths were 3.00–5.99 mm.)

Feeding	Temp. (°C.)	Conc. 2B (mg./l.)	No. snails	Growth (mm./day)		
				Median	Mean	Range
I	21	0.0	12	0.11	0.10 ± 0.015	0.05–0.13
		0.1	12	0.12	0.11 ± 0.020	0.08–0.19
		0.5	12	0.10	0.12 ± 0.018	0.01–0.28
		1.0	12	0.01	0.02 ± 0.005	0.00–0.08
II	26	0.0	55	0.25	0.27 ± 0.010	0.07–0.38
		0.1	17	0.21	0.22 ± 0.013	0.12–0.30
		0.2	15	0.17	0.18 ± 0.013	0.06–0.26
		0.5	49	0.05	0.07 ± 0.005	0.00–0.14
		1.0	14	0.00	0.00 ± 0.001	0.00–0.02

snails at all times. The rate of growth of snails used in control experiments at 26° has been compared for three feeding methods with the results indicated in Table I.

In all experiments subsequently presented in this report feeding was by method III which gave most rapid growth, unless otherwise indicated, and data are for growth periods during which the median lengths of the snails were from 3.00 to 5.99 mm.

The effect of 2B on growth was quite different from that of the other four drugs and so is presented separately in Table II. Additional information on the effects of 2B were obtained in preliminary experiments conducted at *ca.* 21°. In these experiments, of 27 snails exposed to concentrations of 2B from 2.0 to 10.0

TABLE III

Comparison of growth rates of Physa heterostropha treated with concentrations of 2A, TS, BS and SA showing maximum inhibition of growth with untreated controls. Data for periods in which median lengths of snails were within the range 3.00–5.99 mm.

Feeding	Temp.* (°C.)	Drug	Drug conc. (mg./l.)	No. snails	Mean growth (mm./day)	Per cent inhibition	<i>p</i> **
III	21	none	0.0	16	0.23 ± 0.014	35	<0.00003
		2A	0.5–15.0	25	0.15 ± 0.009		
II	26	none	0.0	55	0.27 ± 0.010	44	0.0013
		2A	2.0–5.0	65	0.15 ± 0.007		
III	26	none	0.0	30	0.41 ± 0.014	37	<0.00003
		BS	50	15	0.26 ± 0.014		
		TS	50	16	0.26 ± 0.016		
		SA	10.0–50	30	0.28 ± 0.015		

* Temperatures varied within approximately two degree ranges.

** Values for *p* have been determined using the Mann-Whitney U test for comparing the data for control and experimental groups (Mann and Whitney, 1947).

mg. per liter, 24 died within two days and the remaining three within four days. At concentrations of 0.5 and 1.0 mg. per liter, *ca.* 50% mortality occurred within four days. One of six died between the tenth and twelfth day in 0.25 PPM 2B and none of 15 maintained as controls or in 0.05 PPM 2B died within 12 days. Mortality was lower at 26°, 4 of 87 snails dying within 10 days in 2B solutions of concentrations 0.5 and 1.0 mg. per liter. Among 55 controls and 32 snails in 0.1 or 0.2 PPM 2B, none died within 10 days.

The four remaining drugs when used with snails growing at the more rapid rates (feeding methods II and III) showed maximum inhibition of growth to comparable extents. The results of these experiments are presented in Table III. The data for concentrations showing maximum inhibition have been examined in classes (as indicated above for control experiments) using the Kruskal-Wallis test and found to be statistically homogeneous (p ranges from 0.1 to 0.5). Data for BS, TS and SA obtained in simultaneous observations have been similarly compared as a group with their controls. The value of p thus obtained was < 0.00003 .

A series of experiments using Diamox was run to compare inhibition of growth of slowly growing snails with that of rapidly growing ones. Mean growth rates

TABLE IV

Growth rates of snails subjected to various concentrations of Diamox and fed for slow growth (Feeding I) at 26° C.

Conc. Diamox	0.0	0.1	0.2	0.3	0.4	0.5	1.0	1.5
Mean growth rate (mm./day)	0.10	0.12	0.10	0.07	0.10	0.08	0.10	0.10
S.E. of mean	0.015	0.019	0.016	0.013	0.016	0.011	0.014	0.016

observed are shown in Table IV. When these control and experimental groups are compared, no statistically significant differences between the growth rates of the groups of snails are found ($p = 0.3$, using the Kruskal-Wallis test).

DISCUSSION

The effect of 2B on the snail *Physa heterostroph*a is quite different from that of the other drugs studied and is of a nature which indicates its action on the snail is not simply one of inhibition of carbonic anhydrase. This result agrees well with the conclusion of Wilbur and Jodrey (1955) that this drug has an effect on calcium deposition in the oyster that is independent of its inhibition of carbonic anhydrase and in their experiments was shown to be independent of the mantle.

The results of experiments using Diamox, BS, TS, and SA with snails fed for rapid growth are consistent with the hypothesis that rapid shell growth in this mollusc depends upon the activity of carbonic anhydrase and it is unlikely that such congruence of effects would occur if the four drugs were acting on different enzyme systems. At low growth rates induced by feeding method I, Diamox did not further decrease growth rates, indicating that under at least certain circumstances of slow growth carbonic anhydrase activity is insignificant in shell growth

of the snail. Such a result does not, however, preclude the possibility that the enzyme may have increased importance in determining the rate of growth under other circumstances limiting shell development, as low temperatures.

The results here reported are entirely consistent with the hypothesis that under certain circumstances carbonic anhydrase is essential for rapid shell development in the snail used but that in some conditions the enzyme is not necessary for shell growth.

SUMMARY

1. The rate of growth of *Physa heterostropha* may be controlled in the laboratory by using different modes of feeding.

2. Under the conditions of the experiments, growth rates for each feeding method, with the possible exception of that giving slowest growth, are constant over the range 3.00 to 5.99 mm. median shell lengths during two-day growth periods but are slower at both higher and lower lengths.

3. The drug 2-benzothiazolesulfonamide completely inhibits growth of the snail at concentrations about 1 mg. per liter. At 21° C. it is lethal at concentrations above this but appears to be somewhat less toxic at 26° C. Its effect is not due to the action of the drug as a carbonic anhydrase inhibitor but to an unknown side effect.

4. Diamox, benzenesulfanilamide, p-toluenesulfonamide and sulfanilamide, at the concentrations exhibiting maximum effect on growth, exhibit comparable degrees of inhibition of shell growth in snails fed for rapid growth.

5. Diamox at concentrations up to 1.5 mg. per liter does not decrease the growth rate of snails induced to grow slowly by the feeding method employed though this concentration inhibited growth to a significant degree in rapidly growing snails.

6. The results are consistent with the hypothesis that carbonic anhydrase is essential in rapid shell development of some molluscs but may be insignificant in slow growth induced by poor feeding conditions, and afford additional evidence that the enzyme is significant in mollusc shell formation.

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THE PHYSIOLOGY OF SKELETON FORMATION IN CORALS. III.
CALCIFICATION RATE AS A FUNCTION OF COLONY WEIGHT
AND TOTAL NITROGEN CONTENT IN THE REEF CORAL
MANICINA AREOLATA (LINNAEUS)

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This paper deals with an experimental study of the relationship between colony size, total nitrogen content, surface area and rate of calcification in the reef-building coral *Manicina areolata* (Linnaeus).

Tamura and Hada (1932) stated that coral growth ceases after certain colony sizes are attained. The Stephensons (1933) reported that the percentage annual increase in surface area and diameter was much greater in small colonies than in large ones. No attempt was made by these authors to determine whether the faster growth rate of younger corals was due to some inherent physiological factor, or whether it was due to the increasing disproportion of skeletal mineral matter, *e.g.* volume, in relation to the surface area occupied by the polypal mass, as corals get larger and older. Abe (1937) found that freshly settled larvae of *Fungia actiniformis* grew about three times faster between the first and fifth than from the sixth to the sixteenth days. Motoda (1940) believed that the decrease in the growth rate of the reef-building coral *Goniastrea aspera* with increase in colony diameter was the result of physiological senescence, and the progressive restriction of surface area.

To investigate the relationship of colony size on total nitrogen content and on the calcification rate in a reef coral, we chose *Manicina areolata* as the experimental animal. This species is plentiful on nearby reefs. Colonies of all sizes were obtained in numbers and even the largest were small enough to fit conveniently into our experimental vessels. *Manicina areolata* is found on the coral sand, shingle and grass bottom environments which are associated with the reef tract south of the town of Port Royal, Jamaica. This species is unique among West Indian reef-building Scleractinia in that it spends only a relatively short initial part of its life cycle attached by a stalk to small bits of shingle, shells or other corals. On reaching weights of approximately ten grams, the colonies usually break free, probably by accident, and fall unattached to the bottom where they continue to grow and proliferate.

PROCEDURE

Colonies of *Manicina areolata*, weighing between 0.047 and 148.5 grams wet, were collected on 11 May 1959 from the lagoon of Drunkenman's Cay, without

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exposing them to contact with air. These were taken to the laboratory and kept for two days in large, well illuminated tanks provided with fresh circulating sea water to acclimatise them to their new environment. Colonies showing obvious damage, infestations of *Pyrgoma*, worms or other boring animals were rejected. To avoid injury, those of the smaller colonies which were still growing on pieces of rock or shell were not removed from their attachments.

After acclimatisation, the colonies were transferred, still under water, into large polyethylene vessels which were calibrated to contain three and a half liters. To this volume of unfiltered sea water was added an amount of buffered $\text{Ca}^{45}\text{CL}_2^2$ sufficient to give a specific activity of about 10^4 counts per minute per milligram of calcium in the aquatic medium. Illumination was provided by a twin bank of 40-watt daylight fluorescent tubes located about 12 inches above the water level. The water was stirred and aerated by a fine stream of wet air forced through a sintered glass filter stick which served as airstone. The pH of the medium increased from 8.20 to 8.34 during the experimental runs, and the water temperature fluctuated between 29.3°C . and 27.5°C . The experiments were allowed to run for 50 hours, water samples being taken after one, 24 and 48 hours for the measurement of the specific activity.

The runs were terminated by transferring the corals from the Ca^{45} -enriched water into fresh "cold" running sea water for two hours to allow washing out of all activity not deposited into the skeleton. The specimens were killed by putting them into a solution of slightly alkaline 4% formaldehyde. Later, the corals were placed on filter paper to remove the adherent fixative. Algae and other encrusting organisms were carefully scraped away from the non-living base of each colony, and the length, width and weight were recorded for individual specimens. The colonies were then dissolved in separate flasks containing concentrated HCl, the volume of which was adjusted to the weight of the coral. After solution of the mineral matter the tissue was homogenised with a Potter grinder, and the final volume of the suspension carefully determined. The total nitrogen contained in each colony was calculated from replicate 3-ml. aliquots treated according to the Kjeldahl method.

The weight of calcium laid down during the experiment by each specimen was determined from the amount of calcium⁴⁵ incorporated and the mean specific activity of the water in which the corals were run, according to the method described in a previous paper of this series (Goreau, 1959a). The final results were expressed in terms of weight of calcium deposited per gram of wet weight, *e.g.* mg. Ca gm^{-1} , or as the amount of calcium deposited per milligram of nitrogen, *e.g.* mg. Ca mg. N⁻¹. The latter will be called the "specific calcification rate," and the use of the term "growth" in this paper is taken to mean increase in weight due to calcium carbonate deposition.

Besides using normal colonies of *M. areolata* containing large numbers of zooxanthellae, experiments were run concurrently, and under identical conditions, on a series of five colonies which had previously been grown in darkness for three months to cause complete loss of the zooxanthellae and boring algae. The coenosarc of these specimens was colourless and quite translucent in the expanded condition.

² Supplied as Ca-45-P-2 by the Oak Ridge National Laboratory.

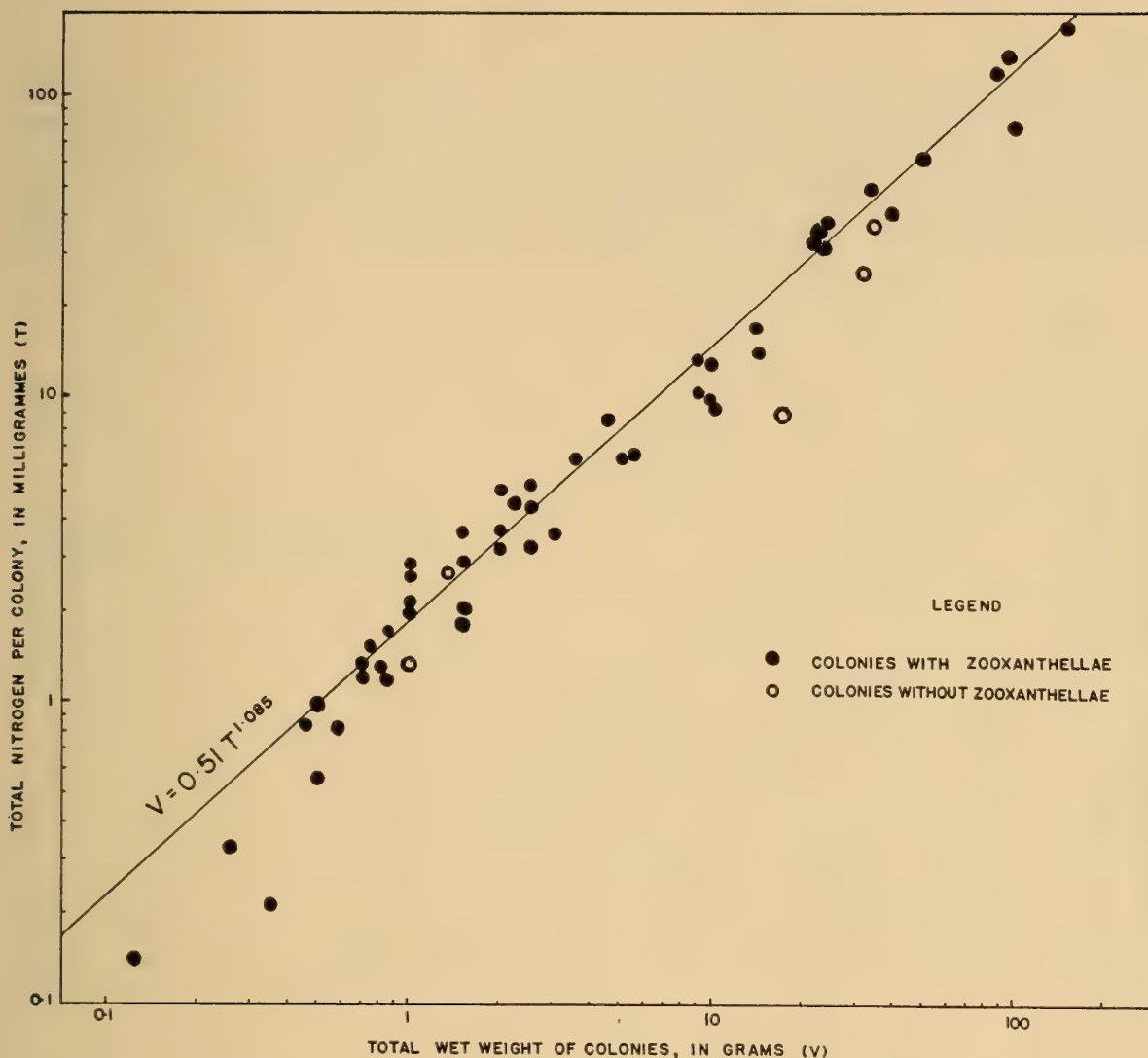


FIGURE 1. The relation between wet weight and total nitrogen content in colonies of the reef coral *Manicina areolata*. The solid circles represent colonies with zooxanthellae, the open circles are from colonies lacking zooxanthellae. The regression line was calculated by the method of least squares using the data from normal colonies only. The slope of the line is very close to unity, thus indicating that the volume, as measured by the weight, and the surface area, as measured by the total nitrogen, increase at the same rate. The significance of this is explained in the text. The position of the open circles near the regression line indicates that in comparison with normal colonies of the same weight, the nitrogen content of the bleached colonies is not significantly reduced by the absence of both zooxanthellae and boring algae.

In one colony a faint green discolouration was seen in the skeleton, indicating that some boring algae were still present although zooxanthellae were absent.

Dead colonies of *M. areolata* of different sizes, from which all tissues had been removed by maceration, were run in each series to test the amount of equilibrium exchange of calcium between the bare skeleton and the aquatic medium. No corrections were necessary for exchange since this occurred at an extremely low level in comparison with active deposition of calcium by living colonies. There was also no correlation between equilibrium exchange rates and the weight of the colonies used.

RESULTS AND OBSERVATIONS

We found that the total nitrogen content of specimens of *Manicina areolata* was directly related to their wet weight over the range of sizes investigated. This is shown in Figure 1 where the wet weight of the corals is plotted against their total nitrogen content. Logarithmic scales are used because of the large numerical range of the values. The nitrogen content of the smallest colonies tested

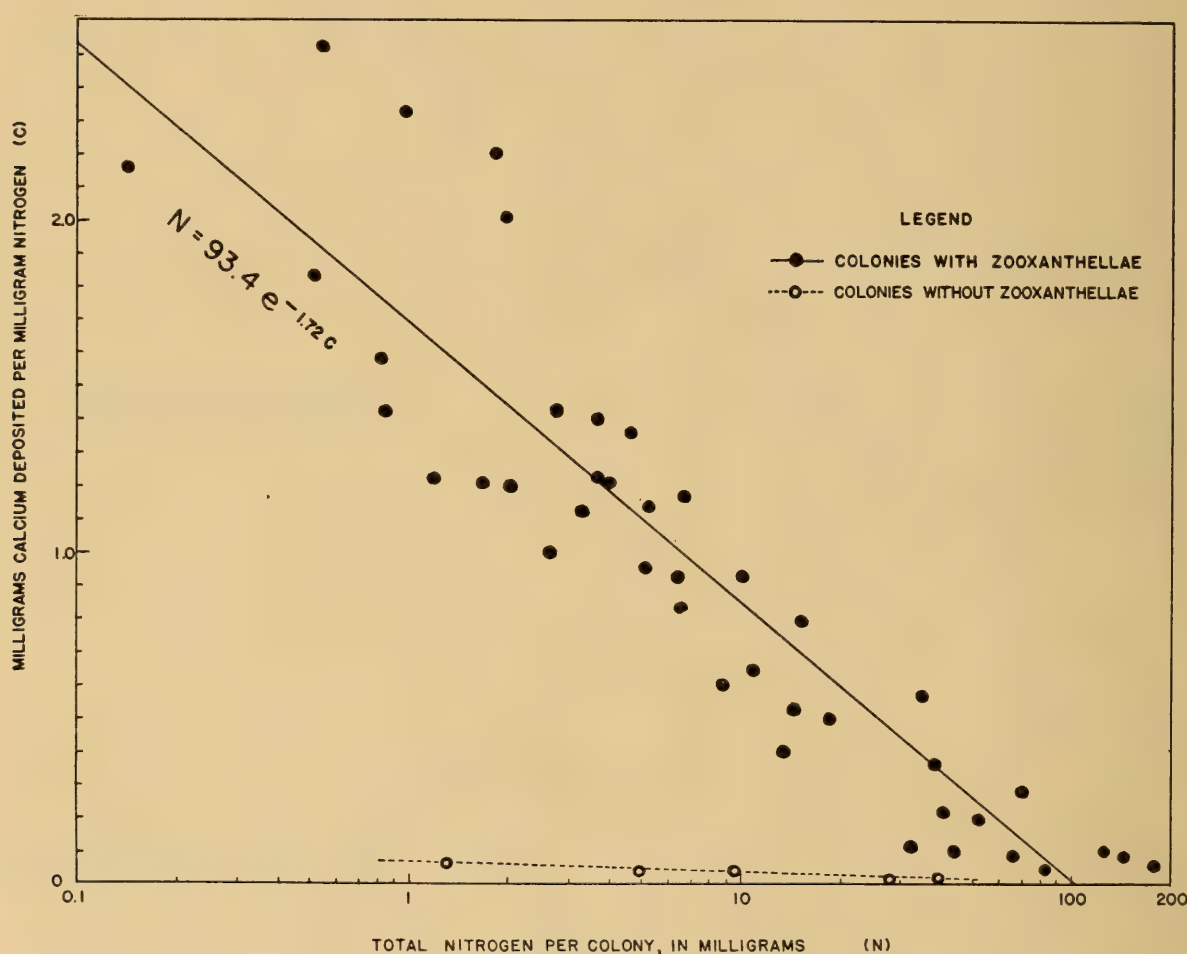


FIGURE 2. Graph showing the relation between the specific calcification rate and the total nitrogen in colonies of *Manicina areolata*. Corals with zooxanthellae are represented by solid circles, the bleached corals by open circles. The slope of the solid regression line was calculated by the method of least squares whereas the lower broken line was fitted by eye. These curves show that calcium is deposited at a progressively slower rate as the size of the colonies increases. The specific calcification rate of corals with zooxanthellae was about 30 times higher than that of bleached colonies of similar size.

was too low to determine by our Kjeldahl method; hence data from these were omitted from Figures 1 and 2.

The position of the points in Figure 1 indicates a good linear correlation between the wet weight V and the total nitrogen T . The regression line was fitted by the method of least squares and yields the equation,

$$V = 0.51 T^{1.085} \quad (1)$$

for the relation between V and T . The power of T is very nearly unity, which indicates that the ratio of wet weight to the total nitrogen is more or less constant over a large range of colony sizes. This means that, in individuals of *M. areolata* weighing between 0.1 and 148.5 grams, the amount of living tissue remains approximately the same in proportion to the total mass of skeletal calcium carbonate.

In colonies lacking the zooxanthellae, the relation between the weight of the individual specimens and their total nitrogen content does not appear to differ significantly from that of normal colonies of similar weight, as shown by the data plotted in Figure 1. Our results for *M. areolata* conflict with the assertion by Odum and Odum (1955) that the average living reef coral colony contains three times as much plant matter as animal matter. Bleached *Manicina areolata*, which lacked both zooxanthellae and boring algae, did not show the expected decrease in the protein nitrogen to weight ratio as compared with normal colonies of the same size. If these corals were to contain as much plant matter as stated by the Odums, then the nitrogen content of the bleached and zooxanthella-less corals should have been much less, size for size, than that of normal colonies. The fact that it was nearly the same would imply that the aggregate plant biomass associated with *M. areolata* is very much less than that found by the Odums for other species of corals.

With increasing size, we observed a striking fall in the calcification rate. The smallest colony tested, weighing 0.047 gram wet, deposited 92 mg. Ca gm. coral⁻¹ hr.⁻¹ whereas the largest, weighing 148.5 grams wet, deposited only 1.28 mg. Ca gm. coral⁻¹ hr.⁻¹. This is a 76-fold difference. The above calcification rates are given in terms of weight rather than nitrogen because the smallest colonies used contained too little nitrogen to be reliably measured by our Kjeldahl method.

On the whole, the relationship between the specific calcification rate C to the total colony nitrogen content N , as plotted in Figure 2, is given by the equation,

$$N = 93.4 e^{-1.72 C} \quad (2)$$

whereas the relation between calcification rate per gram of coral W to the total wet weight V , not shown in the figure, is given by the equation,

$$V = 63.8 e^{-1.55 W} \quad (3)$$

These formulae show that the growth rate, as expressed in terms of the rate of calcium deposition, decays as an exponential function with increase in size. Similar but less marked trends were observed in colonies lacking zooxanthellae, but in these the calcification rate, size for size, was 30 times less than that of normal colonies of *M. areolata*.

The intercept of the regression line with the x-axis in Figure 2 implies that colonies having more than about 102 mg. total nitrogen do not grow any more. There is no evidence for this, and the decremental relationship cited above may hold only for colonies below a certain size range. Our results show that much larger colonies still grow slowly, but we do not yet have enough data to determine what kind of relationship, if any, may be applied to describe growth versus size in such cases.

For corresponding colony sizes, the calcification rates observed in these experiments on *M. areolata* with zooxanthellae were similar to those previously re-

ported for this species under field conditions (Goreau and Goreau, 1959), but growth rates of the zooxanthella-less specimens were lower by a factor of about 1.5. A possible reason for the discrepancy is that the bleached *M. areolata* used in the present experimental series had been grown in laboratory tanks in darkness for three months whereas those used in our previous study were found growing in the decolourised state under a coral head in the natural environment of the reef. Under these conditions, the growth rate of the naturally bleached colonies may have been faster because of their better nutritional status.

DISCUSSION

In the coral *Manicina areolata* there is an approximate one to one relationship between the wet weight of whole colonies and their protein nitrogen content, and the specific calcification rate is inversely related to the weight, *i.e.*, size, of the individual corals.

The linear relationship between colony mass and the amount of protein nitrogen in specimens, with and without zooxanthellae, is extremely interesting. Except for the boring algae, all living tissue in scleractinian corals occurs outside the surface of the skeleton. Therefore, the amount of nitrogen per colony will be a function of the total surface area covered by the living polyps. In massive hemispheroidal corals, an increase in the colony size causes the weight, *i.e.*, mass of skeletal calcium carbonate, to increase in proportion to the cube of the radius, whereas the tissue mass, *i.e.*, the surface area, increases only in proportion to the square of the radius. The relationship, which has been demonstrated by Kawaguti (1941) for the massive coral *Goniastrea aspera*, does not hold for *M. areolata* where the volume and surface area increase in direct proportion to each other. Inspection of *M. areolata* colonies of various sizes shows why this is so. Very small young individuals are shaped like simple cups. These grow in size and proliferate by incomplete intratentacular budding. This results in a lengthening of the tentacular ring and oral disk, along with an increase in the number of the stomodea. At first, this growth produces a simple linear elongation of the colony until the length is about twice the width. As growth continues with the budding of new stomodea, the valley becomes sinuous and the edge of the theca folds so that the whole colony presents a convoluted appearance which becomes increasingly complex as the coral grows larger. A typical growth series is shown in Figure 3. In this manner, the area, as measured by the protein nitrogen, and the volume, as measured by the weight of calcium carbonate, retain a one to one relationship to each other, as demonstrated by the slope of the regression line in Figure 1.

Such a large increase in the surface area relative to the weight is almost certainly adaptive response to the ecological conditions in which *M. areolata* lives. This coral is very common in shallow water; the larger colonies are usually found lying loose on sand or shingle bottom, frequently in association with the marine grass *Thalassia* (Goreau, 1959b). Our reef surveys have now shown that it is also found in large numbers on the sand and rubble bottom of canyons in the reef buttress zone at depths down to at least sixty feet where it is one of the few corals which successfully and consistently colonise this particular habitat (Goreau, unpublished field observations). It is therefore clear from its ecology that *M.*

areolata is adapted for life on unstable substrates where it is constantly exposed to the danger of burial by shifting sediments, or to being overturned by wave turbulence and currents. The species has a very efficient mechanism for ridding itself of sediment by means of flagellar currents and moving sheets of mucus. It is also one of the few corals possessing a well developed righting reaction. We have observed colonies weighing up to about 65 grams successfully right themselves after having been turned upside down. This was accomplished by a great swelling

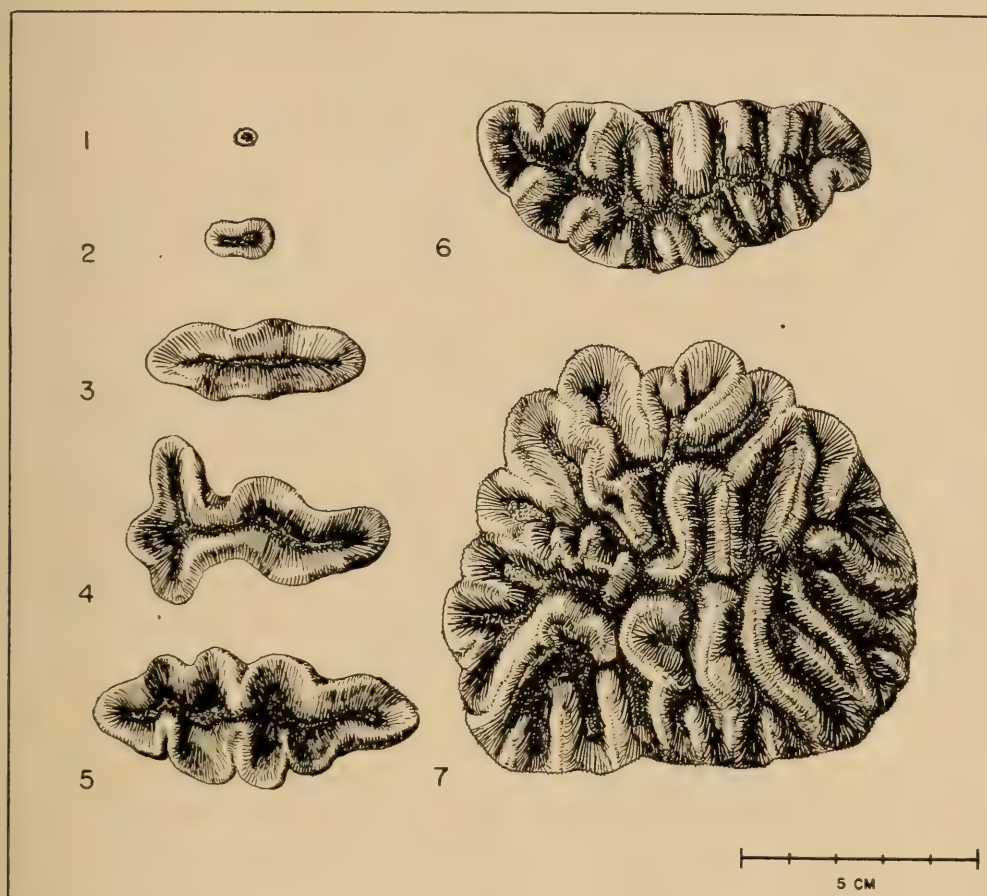


FIGURE 3. Relation between size and development of surface convolutions in a growth series of the coral *Manicina areolata*. The dry weights of these specimens are 0.09 gm., 0.74 gm., 6.2 gm., 10.3 gm., 21.4 gm., 58.7 gm., and 117 gm., respectively. The smallest individual shown here has a simple cup shape and only one stomodeum. Proliferation of additional stomodea by intratentacular budding at first produces linear elongation of the theca; later this becomes gradually more complex by formation of folds and branchings. Eventually, the typical convoluted brain-like surface pattern seen in the largest specimen is achieved. The continuous valleys of the specimens shown indicate that they are all clones descended from single planulae. Colonies of *M. areolata* which are formed through fusion of more than one planula invariably have a corresponding number of separate and distinct valley systems.

of the coenosarc through imbibition of water which was then vigorously ejected from the stomodea in the form of jets directed against the sand, thus undermining the colony on one side and rocking it until it was gradually righted in the normal position (Goreau, unpublished observations). It is almost certain that the ability of this coral to cleanse itself of sediment and to right itself if overturned must depend on its having a large surface area with respect to mass.

An interesting parallel is offered by the fungiid corals of the Indo-Pacific, which occupy an ecological niche in reefs very similar to that inhabited by *M. areolata* in the Western Atlantic, and which show striking analogies in their adaptive specialisations even though these corals belong to a different suborder of the Scleractinia. The young stages of both are attached to the substrate by stalks which subsequently break so that the older colonies are usually found free. In the fungiids, the stalk continuously buds off new colonies, in *Manicina* it does not. Both corals are commonly expanded in the day time; both increase their surface areas in more or less direct proportion to their mass, the fungiids doing this by assuming a flattened disk-like shape, *Manicina* by becoming more and more convoluted as its size increases. Both corals can inhabit environments where sedimentation and substrate instability prevent colonisation by most other species, largely through their innate ability to remain "afloat" on top of sand and shingle. In both, the highly sensitive youthful stages remain attached until they are heavy enough to resist displacement and turning over by anything but the strongest waves and currents.

Unlike the surface area, the specific calcification rate of *M. areolata* decreases with an increase in colony size. Motoda (1940, quoted by Wells, 1957) studied growth in *Goniastrea aspera* and concluded that progressive restriction of the surface area causes crowding of polyps in the center of the colony, thus slowing the growth rate as the spherical form is approached. We do not believe that these factors operate in the case of *M. areolata* since the surface area of this coral increases in direct proportion to the size. All colonies used in our experiments were descendants of single planulae as shown by their continuous valley patterns. There was no evidence that the stomodea in the center of the colonies were more crowded than those of the periphery.

Of all other variables which could influence the calcification rate with increasing age, the most obvious are the zooxanthellae. These dinophycean algae occur in the gastroderm of all hermatypic corals as intracellular commensals, and are known to affect the metabolism and growth rates of their hosts in a number of important ways. The presence of photosynthesising zooxanthellae constitutes a most effective stimulus to the mechanism of calcification, causing a very large increase in its rate in all species of reef corals we have tested so far (Goreau and Goreau, 1959). This effect is probably mediated by a decrease in the HCO_3^- concentration at the site of calcification when the zooxanthellae are photosynthesising, *i.e.*, removing CO_2 from the system (Goreau, 1959a). As is shown by the data given above, the calcification rate of *M. areolata* without zooxanthellae falls to extremely low levels in comparison to colonies of the same size which contain the zooxanthellae; nevertheless the growth rate of the smallest of our bleached colonies was nearly the same as that of the largest of the normal colonies that were tested. There is no evidence that the low calcification rate we found in the large specimens was due to a systematic reduction in the number of zooxanthellae with increasing age or size. On the contrary, large colonies of *M. areolata* are usually a darker brownish green than the young colonies which are mostly pale greenish brown in colour. Histological cross-sections of the oral disk show that there are about half as many zooxanthellae in the average thickness of the gastrodermis of small colonies about one centimeter long than in larger colonies about ten centimeters long. This means that the younger smaller colonies seem to have

fewer zooxanthellae per unit of weight than older larger colonies. The precise nature of this relationship is now being investigated in our laboratory. The decrease in calcification rate with increasing size is perhaps not due primarily to any variation in the number of zooxanthellae, but it occurs independently, as shown by our data on corals without zooxanthellae, in which this effect was also observed though it was much reduced in amplitude.

The mechanism by which age affects growth and other metabolic processes (*cf.* Brody, 1945) is not well understood. Although the possibility is suggested by our experimental results on *M. areolata*, it is still not clear whether other corals undergo a true process of senescence if all environmental factors remain optimal. There are probably very great individual variations in this respect among the different species of reef-building corals, as is indicated by the enormous variations in the size of some of them. Single colonies weighing several hundred tons are not uncommon. Many coral colonies are clones which are descended from single planulae. In these a progressive reduction in the speed of growth processes would be expected with increasing age of the coral. Similar phenomena have long been recognised, for example, in protozoa such as *Paramecium* and *Uroleptus* (Calkins, 1926) in which clonal ageing results in gradual failure of cell division.

There are almost certainly exceptions to this among the corals. For example, our observations on the branching West Indian staghorn coral, *Acropora cervicornis*, suggest the possibility that there is no reduction in the over-all growth rate with increasing size and/or age. In this species most of the growth takes place in the initial few centimeters of the branch tips occupied by large pale terminal polyps which have a far higher calcification rate than the much smaller lateral polyps immediately behind (Goreau and Goreau, 1959). These fast growing apical polyps are often seen to differentiate apparently in a spontaneous way, giving rise to new branches in parts of colonies which are already comparatively old and where the calcification rate is very low. The ability of these corals to grow new branches whenever and wherever needed must confer upon them an enormous adaptive advantage, since this enables them to respond rapidly to damage by waves and sediment. By using some of the lower branches as stilts to hold the main part of the colony off the bottom, it becomes possible for *A. palmata* to colonise sandy unstable habitats which would normally be inaccessible to most other corals having a more determinate pattern of growth.

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SUMMARY AND CONCLUSIONS

1. A study was made of the relation of colony weight to the total nitrogen content and calcification rate in the reef coral *Manicina areolata* (Linnaeus). Colonies weighing between 0.047 and 148.5 grams were used in our experiments.

2. In *Manicina areolata*, the ratio of the surface area, as measured by the total nitrogen, and the volume, as determined by the weight of the coral, remained nearly constant over the range of colony sizes tested. This is due to the fact that the surface of the coral becomes more and more folded as the colony grows older and larger. In those massive hemispheroidal corals which lack convoluted surfaces the increase in area would occur as a function of the square of the radius whereas the volume would increase as a function of the cube of the radius. It is suggested that the disproportionately large increase in the surface area of *M. areolata* with respect to growth in size, or volume, is an adaptive specialisation which aids this coral to colonise sandy or otherwise unstable bottom substrates, to clear itself more rapidly of sediment by means of flagellar currents, and to right itself if overturned by waves or currents.

3. The specific calcification rate of the smallest colonies was about 76 times greater than that observed in the biggest specimens of our series. The slopes of the plotted curves indicate that the growth rate decays as an exponential function of the size, *i.e.* the age of the coral, which can be described by a relationship of the type $X = Ae^{-\lambda Y}$ where X = total colony size, weight or nitrogen content, and Y = the amount of calcium deposited per unit weight or nitrogen. The growth rate of the largest colonies was somewhat greater than expected, and there is reason to believe that the relationship given above may hold only for colonies weighing less than about 100 grams.

4. Similar but much less marked decreases of the specific calcification rate with size were observed in *M. areolata* colonies which lacked zooxanthellae. It is suggested that, although the zooxanthellae are powerful stimulants to the calcification reaction, they are not as such responsible for the decay of the growth rate with increasing size and/or age.

5. The causes for the reduction in specific calcification rates with age are not understood, but may be due to an effect of senescence, similar in principle to the reduction in cell division which has been found to occur in old protozoan clones. Evidence is presented that some of the branching acroporid corals may not undergo a slowing of growth with age since these are able to sprout new fast growing branches even from the older parts of the colony. The survival value of this is discussed.

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THE ACTION OF VARIOUS GOITROGENS IN INHIBITING LOCALIZATION OF RADIOIODINE IN THE THYROID AND THYMUS GLANDS OF LARVAL TREE TOADS

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Because of the differential affinity of the thyroid gland for iodine, properly adjusted doses of radioactive iodine (I^{131}) can produce enough radiation to destroy the thyroid completely without seriously damaging surrounding tissues. Immersion of tadpoles of *Hyla versicolor* at midlarval stages for 24 hours in a solution containing 20 $\mu\text{c.}/\text{ml.}$ of I^{131} results in rapid radiation damage to the thyroid so that no functional remnants are present after 10 days (Dent and Hunt, 1952). Administration of certain chemical substances known to inhibit the thyroid's ability to concentrate or bind iodine might be expected to prevent the thyroid from receiving enough radiation to cause permanent damage. Rugh (1953) found treatment with thiouracil effective in preventing or decreasing radiation damage in the thyroids of *Triturus pyrrhogaster* given a single injection of I^{131} (25 $\mu\text{c.}$).

Dent and Hunt (1952) observed that, in the tadpole, iodine is bound in other organs, notably the thymus, to a lesser degree than in the thyroid. It was demonstrated by Lynn and Dent (1957) that phenylthiourea inhibits iodine binding in both the thyroid and the thymus.

There are several different types of thyroid inhibitors with diverse modes of action. In the present study we have shown that some of them differ in degree and duration of effect by testing their capacities for preventing or diminishing radiation damage to the thyroid and thymus during administration of a large dose of I^{131} .

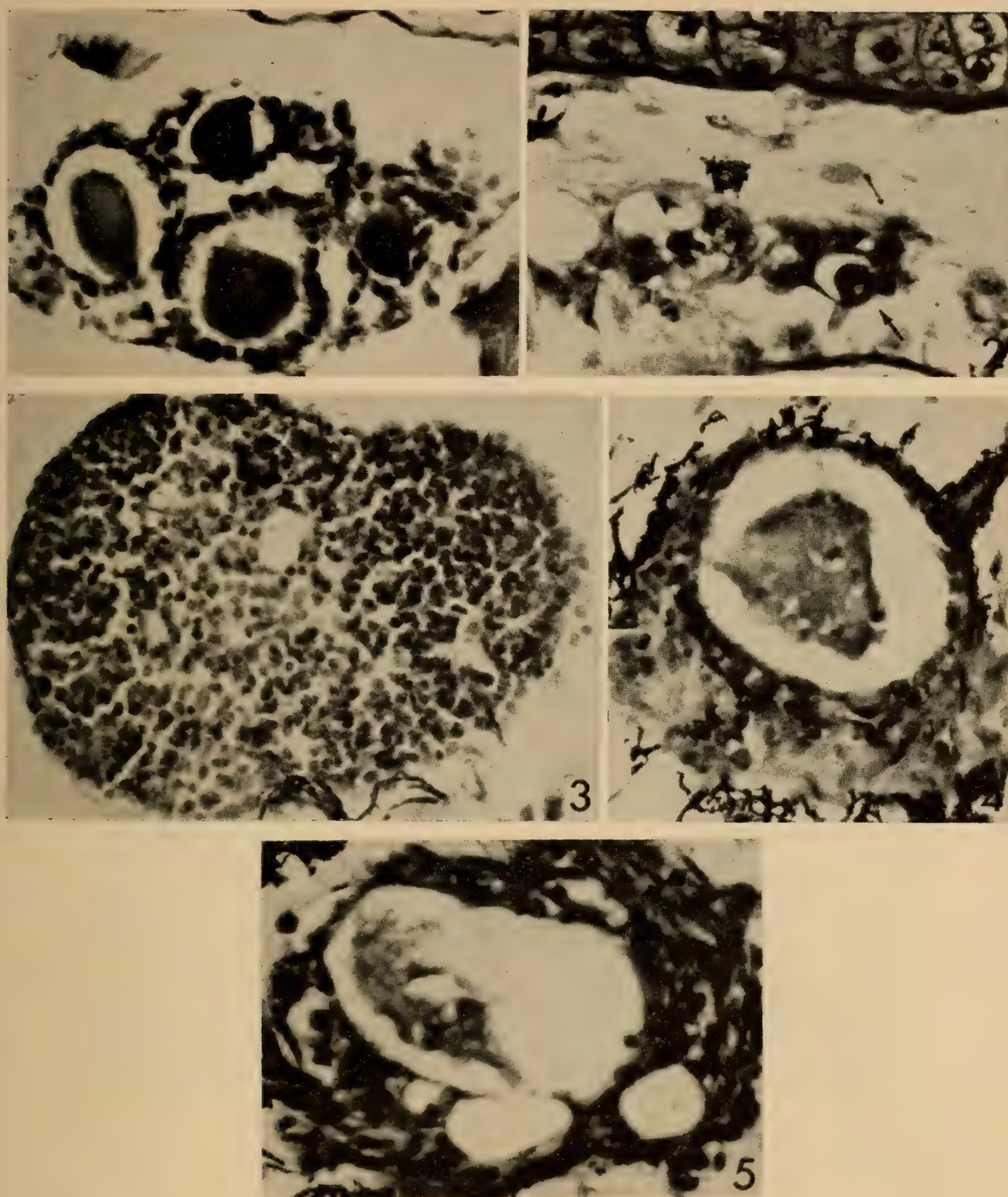
MATERIALS AND METHODS

Tadpoles of *Hyla versicolor* collected near Oak Ridge, Tennessee, were maintained in the laboratory at $21^{\circ} \pm 1^{\circ}$ C. until they reached late limb bud stages (equivalent to stages III to V of the Taylor and Kollros (1946) series). At this time they were divided into control and various experimental groups. Drugs to be tested were dissolved in the spring water in which the experimental animals were raised. The tadpoles were fed crumbled pellets of Purina Rat Chow, an excess of food being available for ~ 8 hours each day. The culture media were changed daily.

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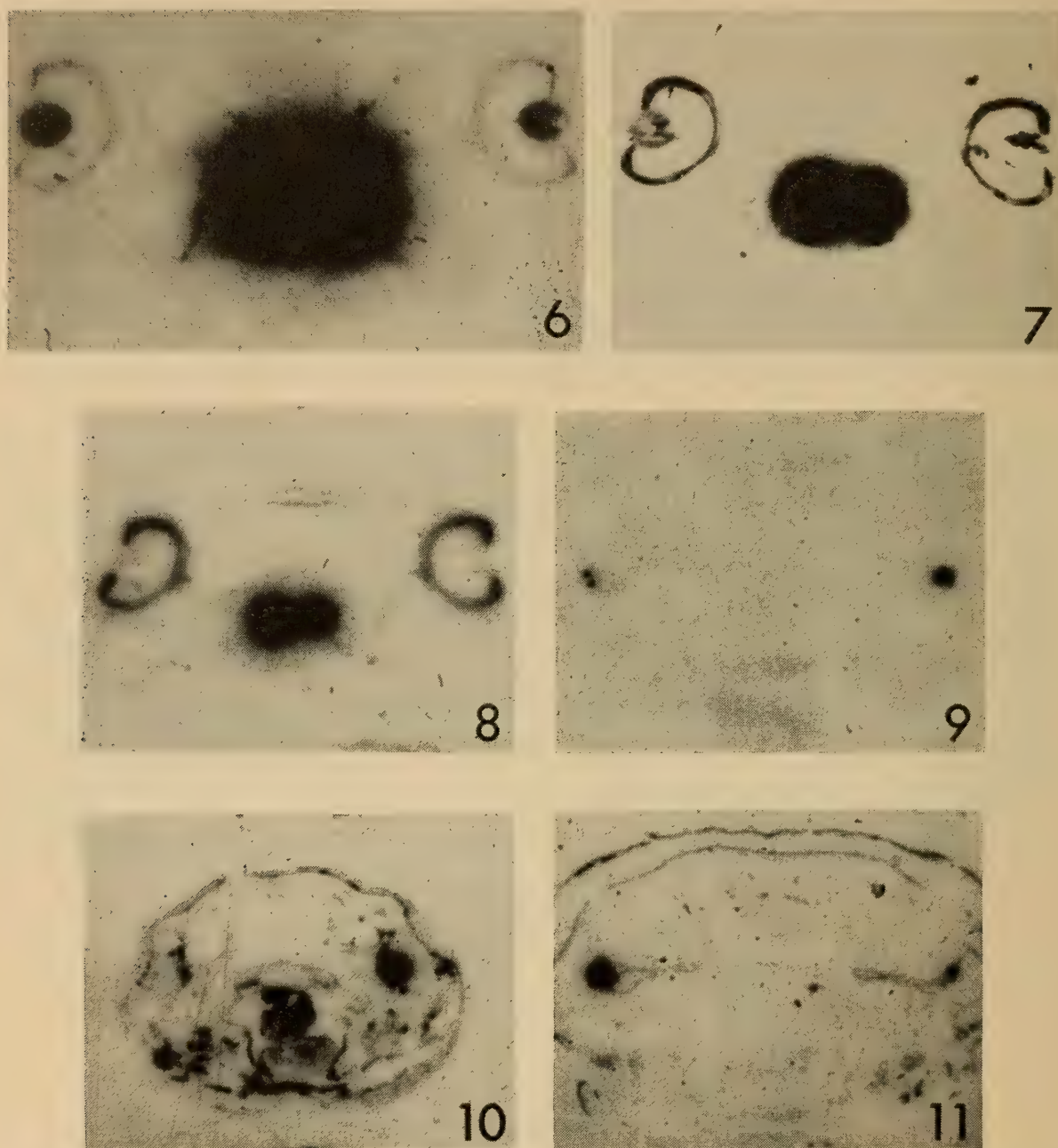
Figures 1 through 5 are photomicrographs of sections of larval specimens of *Hyla versicolor*.

FIGURE 1. Thyroid gland from control specimen with histological evidence of moderate secretory activity.

FIGURE 2. Arrow points to remnant of a thyroid follicle from an animal that received a thyroid-destroying dose of I^{131} and no goitrogen. Thyroid glands from most of the animals treated in this fashion were completely destroyed.

FIGURE 3. Thymus gland from control specimen. Note cords of cells and small sinus.

FIGURES 4 AND 5. Thymus glands showing severe radiation damage. The one in Figure 4 has a single cyst, the one in Figure 5 a large cyst and two smaller ones.



Figures 6 through 11 are photomicrographs of contact autoradiograms made from sections of larval specimens of *Hyla versicolor* treated with I^{131} .

FIGURE 6. Thyroid region of control tadpole after a tracer dose of I^{131} . The spots produced by the two lobes of the thyroid gland are fused into a single spot the size of which is many times that of the gland (compare size of images of eyes).

FIGURE 7. Thyroid protected from the histological damage of the thyroid-destroying dose of I^{131} by goitrogen and then given a tracer dose of I^{131} . The size of the spots indicates that the gland is functioning at a lesser rate than glands of control animals.

FIGURE 8. Thyroid protected from histological damage. No tracer dose was given. The iodine present is residual from the thyroid-destroying dose.

FIGURE 9. Thymus region of control animal given only a tracer dose of I^{131} . The lobes of the thymus have produced the spots on either side of the photograph. They are slightly larger than the sections of the gland that produced them. The double spot on the left represents concentrations of I^{131} in two sinuses. The diffuse spot in the lower center represents the heart.

After a 15-day period⁴ of goitrogen administration one series of tadpoles was given a large (thyroid-destroying) dose of radioiodine by immersion for 24 hours in a solution containing 20 $\mu\text{C.}/\text{ml.}$ of I^{131} . They were then passed through two baths of spring water and maintained in spring water, with the usual daily feeding schedule, for 10 days. These animals and some not previously exposed to radioiodine were given a tracer dose of radioiodine by immersion for 24 hours in a solution of 1 $\mu\text{C.}/\text{ml.}$ of I^{131} . In a second series of animals, the same procedures were followed except that the tracer dose was omitted. All tadpoles were kept in spring water for an additional day and were then fixed in Bouin's fluid, embedded, and sectioned. Contact autoradiograms were prepared after the method of Dent and Hunt (1952) by apposing slides bearing sections of the larvae to Eastman medium-contrast lantern slide plates for an exposure time of 8 days. All sections were subsequently stained with Harris's hematoxylin and Ponceau de xylidine-orange II (Gray, 1952). Figures 1–5 are photomicrographs of sections of larval specimens. Figures 6–11 are photomicrographs of contact autoradiograms made from sections of larvae treated with I^{131} .

RESULTS

1. Thyroid gland

(a) *Effects of radioiodine treatment on normal thyroid.* Tadpoles kept continuously in spring water and given only the tracer dose of I^{131} provided the basic controls in this experiment. Histological observations of thyroid glands from eight such animals indicated moderate secretory activity (Fig. 1). Large follicles were lined with cuboidal epithelium and contained much acidophilic colloid. Chromophobe droplets were present at the periphery of the colloid mass in most follicles. The marked ability of these thyroids to concentrate iodine is evidenced by an intense black spot on the autoradiographic plates over each lobe that fused with the spot representing its companion lobe (Fig. 6).

Administration of the large dose of I^{131} to nine tadpoles resulted in complete destruction of the thyroid in six of them. In the other three, tiny spots running through two or three sections on the autoradiograms corresponded to minute thyroid remnants on the slides. Two of these remnants consisted of isolated colloid masses with no surrounding cells, the other of several follicles with severely damaged epithelia (Fig. 2). These tadpoles, having received no drugs, constitute the experimental controls of the series receiving the thyroid-destroying dose of I^{131} . Table I summarizes the histological findings for all the experimental animals that received the large dose of I^{131} .

⁴ In preliminary experiments, the period of treatment with phenylthiourea varied from 25 to 9 days. Within these limits the protective effect was not correlated with the period of goitrogen administration. The standard period used in subsequent experiments was 15 days.

FIGURE 10. Thymus region of animal given both thyroid-destroying and tracer doses of I^{131} . The large, dark spot on the right represents the right thymus; the left is not present in this section.

FIGURE 11. Thymus region of animal given only thyroid-destroying dose. Only a small part of the right thymus is present. The left thymus has produced a large spot, indicating considerable retention of I^{131} .

(b) *Effects of treatment with phenylthiourea.* In experiments performed to test the degree and duration of effect of phenylthiourea three variations in procedure were followed: (1) treatment with 0.01% phenylthiourea was continued during the 24-hour period of I^{131} administration, the I^{131} solution being made up in phenylthiourea solution; (2) treatment with phenylthiourea was stopped at the time of I^{131} administration, the I^{131} solution being made up in spring water; (3) treatment was stopped two days before I^{131} administration.

The thyroids of eight tadpoles given phenylthiourea before and during I^{131} treatment were indistinguishable histologically from those of the basic control tadpoles, although their physiological activity was reduced in that they produced autoradiograms about half the size of those produced by control thyroids (Fig. 7).

When phenylthiourea treatment was discontinued at the time I^{131} administration was begun, the protective value of the treatment was greatly decreased. Thyroids of five animals handled in this manner showed extensive radiation damage with destruction of cells, pyknosis of nuclei, and, in some regions, complete disruption

TABLE I

Degree of radiation damage in thyroid glands of tadpoles after immersion in solutions of goitrogens and subsequent immersion in solutions containing 20 μ c./ml. of I^{131} (thyroids of controls immersed in iodine without goitrogen treatment were destroyed)

Time of removal from goitrogen solution	Drugs in which animals were immersed				
	Phenylthiourea (0.01%)	KClO ₄ (0.01%)	KSCN (0.005%)	KClO ₃ (0.005%)	KIO ₃ (0.005%)
At end of I^{131} administration	0	0	0	0	0
At beginning of I^{131} administration	++	0	++	NT	NT
Two days before I^{131} administration	+++	0	+++	NT	NT

Legend: 0 = No histological evidence of radiation damage.
 ++ = Evidence of extensive radiation damage.
 +++ = Evidence of complete or essentially complete destruction.
 NT = Not tested.

of follicles. Three of these thyroids produced no autoradiographic images; small isolated spots corresponding to less seriously damaged follicles were present in the others.

Discontinuance of phenylthiourea treatment two days before I^{131} administration resulted in complete destruction of the thyroid.

(c) *Effects of treatment with perchlorate.* Three sets of experiments corresponding with those just described for phenylthiourea were carried out with 0.01% KClO₄.

In contrast with phenylthiourea, KClO₄ completely protected the thyroid not only when given during I^{131} administration, but even when discontinued two days earlier. No histological abnormalities were seen in thyroid sections of this series, and autoradiograms showed dense spots representing the thyroids. Like those from thyroids protected by phenylthiourea, the spots were somewhat smaller than in untreated controls but indicated, nevertheless, a relatively high I^{131} content.

Autoradiograms prepared from sections of animals that did not receive the tracer dose of radioiodine still showed small but definite spots for the thyroid (Fig. 8), indicating that a part of the heavy dose of radioiodine was retained in the protected glands.

Treatment with a lower concentration of $KClO_4$ (0.005%) gave similar results, indicating that these two concentrations are equally effective.

(d) *Effects of treatment with thiocyanate.* Although animals were treated with both 0.01% and 0.005% KSCN, the higher concentration was so toxic that only a few animals survived the experiment, and they were discarded. The lower concentration prevented morphological damage to the thyroid when treatment was continued during I^{131} administration. Cessation of treatment at the time I^{131} was given, however, resulted in extensive damage, only a few badly disrupted remnants of the thyroid being discernible. These remnants were sometimes represented by tiny spots on the autoradiograms. Cessation of pretreatment two days before I^{131} administration resulted in complete destruction of the thyroid.

(e) *Effects of treatment with chlorate and iodate.* A single series of experiments was run to test the effects of treatment with 0.005% $KClO_3$ and 0.005% KIO_3 that was continued during I^{131} administration. Under these conditions these substances prevented any visible histological damage to the thyroid. Autoradiograms showed large intense spots representing the glands.

2. Thymus gland

(a) *Effects of radioiodine on the thymus of control animals.* At the stage studied, the thymus gland is made up of loosely defined cords of cells surrounding small sinuses filled with lymphocytes and erythrocytes (Fig. 3) so that the gland presents a relatively compact appearance in section (Speidel, 1926; Dent and Hunt, 1952).

Administration of the large dose of I^{131} to previously untreated animals resulted in extensive damage to the thymus. In most of them the thymus was represented only by a single large vesicle with a few intact cells around the periphery (Fig. 4). Such glands produced much more intense spots on autoradiograms than did the normal thymus (Figs. 9 and 10). In the relatively few instances in which a number of separate vesicles were present (Fig. 5), rather than a single large one, the autoradiographic spots always corresponded to the vesicles. The material in these vesicles thus had a higher I^{131} content than the cells of the thymus.

(b) *Effects of treatment with goitrogens.* For all the preceding treatments discussed, histological and autoradiographic study was made of the thymus as well as the thyroid. Only one treatment proved completely effective in preventing thymus damage under the conditions of these experiments. Treatment with 0.01% $KClO_4$ continued during I^{131} administration prevented cystic change in the thymus in all nine animals treated. Judging from the extent of damage, similar treatment with 0.005% $KClO_4$ or 0.005% KSCN gave partial protection, but 0.005% $KClO_3$, 0.005% KIO_3 and 0.01% phenylthiourea gave none. No evidence of protection was seen in any tadpoles in which goitrogen treatment was discontinued before or at the time of I^{131} administration.

Any large vesicles present in the damaged thymus were represented by intense spots on autoradiograms. Because these spots were present whether or not a

tracer dose of I^{131} was given (Figs. 10 and 11), it is evident that they resulted primarily from residual iodine of the original dose.

DISCUSSION

Work with mammals indicates that, with the exception of phenylthiourea, the goitrogens used in this study interfere with the thyroid's function by inhibiting its ability to concentrate iodide (Wyngaarden *et al.*, 1952). Phenylthiourea, however, permits iodide concentration but prevents its binding to protein (Pitt-Rivers, 1950; Roche and Michel, 1955). Under the experimental conditions of this investigation all these substances protected the thyroid against retaining sufficient I^{131} to cause histological damage to the gland, provided that the goitrogen treatment was continued during the administration of I^{131} , whereas only $KClO_4$ was effective when pretreatment was discontinued either two days before or immediately before I^{131} administration.

Longer periods between the administration of the large I^{131} dose and fixation of the specimens might produce progressive damage since even those thyroids that were considered to have been completely protected retained a large amount of I^{131} 10 days after the thyroid-destroying dose was given. In mice, damage resulting from a low dose (25 μ c.) of I^{131} is not apparent until many months after the iodine has been injected (Dent, Gadsden and Furth, 1955). Our experiments, however, were directed toward testing the relative effectiveness and duration of effect of the various goitrogenic drugs and were not concerned with devising a method for protecting the thyroid from radiation damage. Continuation of the goitrogen treatment for some time after exposure to I^{131} would presumably provide complete protection.

It is also noteworthy that, in a number of the experiments in which the thyroid seemed completely undamaged histologically, autoradiograms nevertheless indicated that the gland's ability to take up I^{131} from the tracer dose was considerably decreased. This may be interpreted as evidence of physiological damage in the absence of histological changes, or it may indicate a residual inhibitory effect of the drug as seen in the rat after the administration of propyl thiouracil (Durbin *et al.*, 1957).

The only similar experiments with an amphibian are those of Rugh (1953), in which adult specimens of *Triturus pyrrhogaster* were given a series of four intraperitoneal injections of a 1% suspension of thiouracil over a period of 3 weeks. These animals and untreated controls then received an intraperitoneal injection of 25 μ c. of I^{131} . The first indications of damage to the thyroid were seen in the controls after one month, and almost complete destruction had occurred by two months. The specimens given thiouracil showed no damage at one month, minor damage at two months, and clear signs of protection even at 7 months. Even more complete protection might have been afforded, Rugh pointed out, by continuing administration of the goitrogen after I^{131} treatment. Although our experiments were undertaken with a different aim and technique, our results are in accord with those of Rugh.

Our observations also confirm the earlier observation of Dent and Hunt (1952) that the amphibian thymus has a differential affinity for I^{131} and that cystic changes

occur in this gland when sufficient I^{131} is administered. The amount of I^{131} taken up by the thymus is apparently quite low compared with that concentrated by the thyroid. I^{131} concentration in cysts of damaged thymus tissue is apparently much higher than in normal cells of the thymus.

The protective action of goitrogenic drugs in the thymus differed from that in the thyroid. Only one treatment (0.01% $KClO_4$ continued during I^{131} administration) completely prevented cystic changes in the thymus. None of the drugs provided any protection for the thymus if their use was discontinued before I^{131} administration. These goitrogens are all believed to interfere with the thyroid's ability to take up iodine from the circulating blood. Since $KClO_4$ was the most effective inhibitor of I^{131} uptake by both thyroid and thymus, the same mechanism may be operative in both cases. The lesser degree of effectiveness of the drugs for the thymus compared with the thyroid may indicate that the thymus is damaged by lower radiation levels than is the thyroid.

Although protective to the thyroid, phenylthiourea proved to be completely without protective value in the thymus. Although phenylthiourea caused a decrease in I^{131} uptake by the thymus (Lynn and Dent, 1957), the present results indicated that this reduction is not sufficient to protect the thymus from damage by a thyroid-destroying dose of I^{131} .

SUMMARY

1. Larvae of *Hyla versicolor* were immersed for 15 days in spring water containing phenylthiourea, $KClO_4$, KSCN, $KClO_3$, or KIO_3 followed by a 24-hour immersion in 20 μ c./ml. of I^{131} , a thyroid-destroying dose.

2. When treatment with any one of these drugs was continued during the period of I^{131} administration, radiation damage to the thyroid was prevented.

3. The inhibitory effect of $KClO_4$ on the thyroid persisted even when treatment was discontinued two days before immersion in the I^{131} solution.

4. The effects of phenylthiourea and KSCN were dissipated more quickly since the thyroid showed extensive damage when treatment was stopped immediately before I^{131} administration and complete destruction when it was stopped two days before.

5. In control animals, the thyroid-destroying dose of I^{131} extensively damaged the thymus gland as well.

6. Of the drugs tested, only $KClO_4$ sufficiently inhibited the I^{131} uptake by the thymus to prevent damage, and only when it was present during the period of exposure to I^{131} .

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INVESTIGATION OF THE FERTILIZATION INHIBITING ACTION OF ARBACIA DERMAL SECRETION¹

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Several investigations (Oshima, 1921; Pequegnat, 1948; Harvey, 1956; Metz, 1959a) have shown that *Arbacia* releases a fertilization inhibiting agent or agents when subjected to conditions of stress. The agent is present in a yellowish fluid called "dermal secretion" (Oshima, 1921) and is probably released from certain pigment-containing cells of the integument (Pequegnat, 1948). Indeed this dermal secretion is probably responsible for the fertilization inhibiting action ascribed to *Arbacia* blood by Lillie (1914).

Unfortunately, little information is as yet available concerning the mechanism by which the dermal secretion inhibits fertilization. In a previous study (Metz, 1959a) it was found that the dermal secretion inhibits the agglutination of *Arbacia* sperm by fertilizin, the specific sperm isoagglutinin obtained from the jelly layer surrounding eggs. This agglutination inhibiting action was found to result from an inactivation of the specific combining sites of the fertilizin that react with the antifertilizin of the sperm surface.

The parallel inhibiting action of dermal secretion on fertilizin agglutination of sperm and on fertilization suggests that the latter action may also result from inactivation of fertilizin combining sites. The present study was undertaken to investigate the mechanism of fertilization inhibition by the dermal secretion and particularly to determine if this action resulted from an inactivation of fertilizin. The experiments have been reported briefly elsewhere (Metz, 1959b). They indicate that fertilizin is not the site of inhibitor action.

MATERIALS AND METHODS

Two species of sea urchins were used in the experiments, namely *Lytechinus variegatus* and *Arbacia punctulata*. Animals of the former species were obtained from the large population in the vicinity of the Florida State University Marine Laboratory, Alligator Point, Florida. The latter (*Arbacia*) were also obtained from this area, and from Panama City, Florida. This species was also obtained from the Woods Hole, Massachusetts, area and used at the Marine Biological Laboratory.

Gametes were obtained from *Lytechinus* by inducing the animals to shed by treatment with 0.5 M KCl. Gametes were obtained from *Arbacia* by electrical stimulation.

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Solutions of dermal secretion were obtained by immersing *Arbacia* in tap or distilled water for 1–3 minutes, rinsing them in sea water and collecting the yellowish fluid that drained from the animals (Metz, 1959a). The pH of such dermal secretion is about 7.5. The pH was adjusted to that of sea water (8.0–8.2) before use.

RESULTS

In the presence of *Arbacia* dermal secretion both *Arbacia* and *Lytechinus* eggs fail to fertilize, even though these eggs are bombarded by highly motile spermatozoa. Such eggs fail to elevate fertilization membranes or show any indication of nuclear activity. Clearly, then, the dermal secretion blocks fertilization at its initial stages. The first step in analyzing the mechanism of this action of the

TABLE I
Percentage of eggs cleaving after insemination with Arbacia sperm washed from dermal secretion

Dilution of treated sperm	Dermal secretion—treated sperm		S.W.—treated sperm	
	Washed in S.W.	Re-suspended in D.S. supernatant	Washed in S.W.	Re-suspended in S.W. supernatant
1	100% (200 ⁺)	60% (103)	100% (200 ⁺)	100% (200 ⁺)
1/5	100% (200 ⁺)	100% (200 ⁺)	100% (200 ⁺)	100% (200 ⁺)
1/25	90% (128)	100% (200 ⁺)	99% (200 ⁺)	100% (200 ⁺)
1/125	62% (98)	100% (97)	97% (119)	100% (200 ⁺)
1/625	20% (109)	61% (116)	64% (108)	93% (123)

Figures in parenthesis are the number of eggs counted. The four mixtures were prepared in the following proportions: 0.5 ml. approximately 20% sperm suspension; 1.5 ml. D.S. or S.W. These mixtures were centrifuged and the sperm suspended in 1.5 ml. S.W. or in the supernatant. This procedure was repeated and all four sperm suspensions were finally diluted with 25 ml. S.W. (sperm “dilution 1” = approximately 0.4% undiluted semen). The suspensions were diluted serially as indicated. Two drops of unfertilized eggs were added to 4 ml. of each sperm dilution. In a control experiment eggs failed to fertilize when inseminated in the D.S. supernatant from the centrifuged sperm suspension.

dermal secretion was to determine if the agent acted upon the sperm, the egg, or both gametes.

Action on the sperm. In the previous studies (Pequegnat, 1948; Metz, 1959a) it was found that the dermal secretion from *Arbacia* markedly enhanced both motility and respiration of *Arbacia* sperm and that these effects were long lasting. Evidently, then, any fertilization inhibiting action on the sperm must result from some subtle effect such as “premature discharge” of the acrosomal filament or blocking of an essential receptor site on the sperm surface.

To test for such action *Arbacia* sperm were treated with dermal secretion, subsequently washed free of the solution by centrifugation and finally tested for fertilizing capacity. As seen in Table I, *Arbacia* sperm washed from dermal secretion are as effective in fertilizing eggs as sperm washed from sea water. Furthermore, dilution of dermal secretion-treated sperm with sea water restores fertilizing capacity. Thus (Table I) the sperm in dermal secretion gave only 60% fertilization at “dilution 1” but yielded 100% fertilization at higher dilutions.

This inhibition at "dilution 1" is attributed to an action of dermal secretion on the eggs. At the higher dilutions the dermal secretion is evidently diluted beyond the minimum inhibitory concentration. Finally, the data suggest that washing, as opposed to re-suspension in the supernatant, reduces the fertilizing capacity of control sperm. This is in accord with Hayashi's (1945) observations on the beneficial effect of seminal plasma on sperm. Results similar to those in Table I were obtained in a second experiment using *Arbacia* sperm and eggs. Comparable results were also obtained in two experiments with *Lytechinus* sperm and eggs using *Arbacia* dermal secretion. However, the results were not as striking in these experiments because the more delicate *Lytechinus* sperm does not survive centrifugation and re-suspension as well as *Arbacia* sperm.

It is evident from these experiments that dermal secretion does not inhibit fertilization by irreversible action on the sperm. Accordingly, attention was directed toward possible effects on the egg.

Action of dermal secretion on the egg. According to Pequegnat (1948) washing in sea water at least partially restores fertilizability to dermal secretion-treated *Arbacia* eggs. Harvey (1956, page 57), on the other hand, states that "it has been my experience that sometimes the eggs are not fertilizable, even after repeated washings with sea water." Observations in the present study indicated that reversibility of inhibition by washing varies from preparation to preparation. Eggs washed from some dermal secretion preparations fertilize readily whereas those from others show varying degrees of reduced fertilizability. This is evidenced in two ways. As seen in Table III, experiment 1, eggs washed from a dermal secretion preparation may approach 100 per cent fertilizability. However, substantially higher sperm concentrations are required to fertilize such eggs than are required for the controls. A second expression of reduced fertilizability is delayed cleavage. In some experiments *Arbacia* eggs washed from dermal secretion and subsequently inseminated fail to cleave synchronously with the controls. In these, first cleavages are asynchronous and delayed one or two divisions as compared to the controls.

These results indicate that the probability of a "successful sperm-egg contact" (Rothschild, 1956) is lower for the treated eggs than for the control eggs under comparable conditions. The reduced probability of a successful sperm-egg contact in turn could result from a correspondingly reduced number of receptor sites on the egg surface. In the case of cleavage delay the treated eggs may not fertilize at the moment of insemination because time is required for a successful sperm-egg contact to be made. The assumption is then that the egg develops at the normal rate and cleaves on schedule following a successful sperm-egg contact. This view is supported at least to the extent that fertilized eggs show no cleavage delay when placed in the dermal secretion as early as one to two minutes following insemination. However, eggs placed in dermal secretion a few seconds after insemination frequently show arrested fertilization membrane elevation and sperm entry (especially clear in *Lytechinus* eggs). These observations suggest an inhibition of the cortical reaction. In any event these experiments show that washing does not readily restore dermal secretion-treated eggs to the control level of fertilizability. Accordingly, it appears likely that the dermal secretion inhibits fertilization by an effect upon fertilizability of the eggs, as opposed to sperm. This view is supported by studies with enzymes as recorded in a later section.

The effect of dermal secretion on the formation of blebs by inseminated oocytes. The observations described above suggest that the dermal secretion inhibits, blocks or otherwise prevents an early step in fertilization. In a further test of this assumption dermal secretion was examined for its effect on the formation of blebs or papillae on oocytes when these cells are inseminated. As is well known (*e.g.*, Rothschild and Swann, 1949) sea urchin oocytes are not fertilizable. However, these cells, when exposed to sperm, form numerous blebs on their surfaces. Formation of such blebs has been attributed to an interaction between the sperm and oocyte surface. To test for the effect of dermal secretion on oocyte bleb formation, suspensions of eggs containing oocytes were placed in dermal secretion solutions, sperm was then added and the mixtures examined for bleb formation and compared with appropriate controls. Many such observations using both *Arbacia* and *Lytechinus* eggs show that in the presence of dermal secretion bleb formation does not result to a significant degree, whereas numerous blebs formed in the control oocytes. Some inhibitory reaction evidently results which prevents interaction of sperm and oocyte or blocks the bleb-forming response mechanism of the oocyte. In any event these observations support the view that the dermal secretion blocks an early step in fertilization.

In a further attempt to localize the site of action of the dermal secretion, the egg surface was subjected to mild dissection and then tested for sensitivity to the inhibiting action of the dermal secretion.

The effect of dermal secretion on fertilizability of acid-treated eggs. The first agent selected for such dissection was acid sea water. As is well known (Tyler, 1948) acid sea water dissolves the jelly surrounding the egg and thereby removes the fertilizin from the egg except possibly for a layer bound to the egg surface. In two experiments *Lytechinus* eggs were exposed to pH 4–5 sea water and in four experiments *Arbacia* eggs were exposed to pH 5 sea water to remove the jelly layer. These eggs were subsequently washed in sea water, treated with dermal secretion and in some cases washed again and tested for fertilizability over a wide range of sperm concentrations. In all experiments the acid-treated eggs showed the same sensitivity to the fertilization inhibiting action of dermal secretion as did control eggs. Table II gives two experiments, one using *Arbacia* eggs and the other *Lytechinus* eggs. In both experiments it is clear that acid-treated and sea water-treated eggs are both sensitive to the fertilization inhibiting action of the dermal secretion. The well known reduction in fertilizing capacity upon removal of jellies from eggs (Tyler, 1948) is not pronounced at the sperm concentrations employed but indications of this effect are seen in the experiment with *Lytechinus* eggs.

Since the sensitivity of eggs to the fertilization inhibiting action of the dermal secretion is not affected by acid treatment, it follows that the site of action of the dermal secretion is not the egg jelly. It is also presumably not the egg fertilizin since fertilizin constitutes the jelly. However, it is possible that a layer of fertilizin remains bound at the egg surface after the acid treatment and that this is an essential layer blocked by the dermal secretion (*e.g.* Tyler, 1948).

The effect of dermal secretion on fertilizability of eggs treated with proteolytic enzymes. Proteolytic enzymes were used to achieve a further mild dissection of the egg surface. As is well known (*e.g.* Tyler and Metz, 1955; Tyler, Monroy and Metz, 1956), eggs treated with proteolytic enzymes lack jellies, they fail to

form normal fertilization membranes, they become polyspermic readily and hybridize more readily than control eggs when inseminated with sperm of foreign species. The following proteolytic enzyme preparations were used: "crystalline protease" (Nutritional Biochemical Corporation) in 0.1% sea water solution; "crystalline trypsin" (Nutritional Biochemical Corporation) in 0.01% sea water solution. Enzymatic action of the trypsin was stopped prior to addition of dermal secretion by washing the eggs in an excess of trypsin inhibitor (ovomucoid, Nutritional Biochemical Corporation, 0.1% solution in sea water; or soybean trypsin inhibitor, Mann Research Corporation, 0.1% in sea water). In all, ten experiments were performed. These included five with *Arbacia* eggs, one using protease and four employing the combination of trypsin and trypsin inhibitor. Experiments

TABLE II

Effect of Arbacia dermal secretion on fertilizability of acid-treated eggs as indicated by per cent cleavage

	Sperm dilution	Acid-treated eggs		S.W.-treated eggs	
		D.S.-treated	S.W.-treated	D.S.-treated	S.W.-treated
Arbacia eggs	1	23% (124)	91% (141)	15% (114)	90% (200 ⁺)
	1/5	5% (158)	97% (107)	2% (148)	90% (200 ⁺)
	1/25	1% (200 ⁺)	90% (152)	1% (200 ⁺)	90% (200 ⁺)
	1/125	0% (200 ⁺)	81% (128)	0% (200 ⁺)	97% (105)
Lytechinus eggs	1	14.5% (124)	88% (148)	19% (114)	96% (119)
	1/6	6% (114)	97% (147)	4.5% (150)	98% (134)
	1/36	0% (200 ⁺)	46% (140)	1% (200 ⁺)	35% (130)
	1/216	1% (200 ⁺)	14% (180)	0% (200 ⁺)	74% (182)

Arbacia eggs: Jellies were removed by acidification to pH 5.4, eggs were then washed twice in 50-ml. samples of S.W., 0.5 ml. eggs was added to 2.4 ml. D.S. or S.W. and finally three-drop samples of these eggs were added to 5-ml. samples of *Arbacia* sperm. Sperm dilution 1 = 0.1%.

Lytechinus eggs: Jellies were removed by acidification of pH 3.9. Eggs were washed twice in S.W., 0.75 ml. eggs was then added to 2 ml. D.S. or S.W. They were subsequently washed twice in S.W. Four drops eggs were added to 5 ml. of *Lytechinus* sperm. Sperm dilution 1 = 0.66%. D.S.-treated eggs at sperm dilution 1 showed high incidence of polyspermy. Many of the uncleaved eggs contained multipolar spindles.

using *Lytechinus* eggs included four applying protease treatment and one using the trypsin-trypsin inhibitor treatment. In all ten of these experiments enzyme-treated eggs were subsequently treated with dermal secretion (or sea water), washed in sea water and inseminated. With this procedure the enzyme-treated eggs fertilized equally readily whether they received the dermal secretion or sea water treatment. Proteolytic enzyme treatment alone, like acid treatment, lowers the fertilizing capacity of the eggs (*e.g.* Tyler and Metz, 1955). In Table III the results of two of the ten experiments, one using *Arbacia* and the other *Lytechinus* eggs, are presented. In both experiments the fertilizability of sea water-treated eggs was markedly reduced by treatment with dermal secretion. Thus in experiment 1 to even approach comparable fertilization (100% of the eggs), over 125 times the control sperm concentration was required to fertilize the dermal secretion-

treated eggs. In experiment 2 the difference in sperm concentration (to achieve 1% fertilization) was greater than 1000-fold. On the other hand no significant difference is seen between the fertilizability of dermal secretion and sea water-treated eggs that had been pretreated with enzyme. Indeed, comparison of the enzyme-treated and sea water-treated eggs shows that on an absolute basis enzyme

TABLE III

Effect of proteolytic enzymes on sensitivity of eggs to the fertilization inhibiting action of Arbacia dermal secretion

	Sperm dilution	Enzyme-treated eggs		S.W.-treated eggs	
		D.S.-treated	S.W.-treated	D.S.-treated	S.W.-treated
Experiment 1 trypsin-trypsin inhibitor-treated <i>Arbacia</i> eggs	1	99% (149)	97% (154)	89% (158)	100% (200 ⁺)
	1/5	98% (151)	68% (187)	91%† (162)	100% (200 ⁺)
	1/25	42% (295)	21% (186)	3% (156)	100% (200 ⁺)
	1/125	8% (199)	5% (187)	<1% (200 ⁺)	100% (200 ⁺)
Experiment 2 Protease-treated <i>Lytechinus</i> eggs	1	94% (107) (82%)*	93% (97) (51%)*	<1% (200 ⁺)	93% (88) (6%)*
	1/6	87% (118) (52%)*	83% (87) (47%)*	0% (200 ⁺)	98% (96) (2%)*
	1/36	81% (131) (13%)*	12% (149) (0%)*	0% (200 ⁺)	65% (113) (<1%)*
	1/216	37% (169) (<1%)*	25% (128) (<1%)*	0% (200 ⁺)	35% (108) (0%)*
	1/1296	14% (133)	1% (200 ⁺)	0% (200 ⁺)	<3% (200 ⁺)

% = Per cent cleavage.

Experiment 1. Two-ml. samples of *Arbacia* eggs were added to 4-ml. samples of S.W. and 0.05% trypsin (in S.W.). After 45 minutes the eggs were washed twice in S.W., 1.5 ml. of the eggs were transferred to 10 ml. 0.1% ovomucoid in S.W. Subsequently the eggs were washed again, samples were treated with D.S. and S.W. and washed. Four drops eggs were added to 5 ml. *Arbacia* sperm dilution series. A duplicate control experiment omitting the ovomucoid treatment gave similar results. Sperm dilution 1 = 0.1% semen. Degree of polyspermy was not recorded in this experiment. Figures in parenthesis are the total number of eggs counted.

† Cleavage was delayed two divisions as compared to controls.

Experiment 2. Five-tenths-ml. samples of unfertilized *Lytechinus* eggs were placed in 5 ml. 0.1% protease solution or S.W. After 89 minutes 1-ml. samples of protease- and S.W.-treated eggs were each added to 1-ml. samples of D.S. and S.W. Twenty minutes later the eggs were washed in S.W. and 4-drop samples were added to 5 ml. *Lytechinus* sperm at the dilutions indicated. Sperm dilution 1 = 16% of undiluted semen, approximately 2×10^8 sperm/ml. in this case.

* Percentage of eggs counted showing the abnormal cleavage characteristic of polyspermy.

pretreatment results in considerable improvement in fertilizability following exposure to dermal secretion. This improvement is evidently limited at the level of the fertilizability of enzyme-treated control eggs.

In the experiments just described the enzyme-treated eggs were washed free of dermal secretion and inseminated in sea water. If the enzyme-treated eggs are

inseminated directly in the dermal secretion they fail to show the marked improvement in fertilizability. In the discussions given below this reservation concerning the loss of dermal secretion sensitivity following trypsin treatment is assumed.

Two possible explanations for the improving action of proteolytic enzyme pretreatment are evident. The enzyme could destroy the activity of the dermal secretion and thereby protect the eggs from this inhibitor, or the enzyme could act directly upon the egg. Even after several washings sufficient enzyme might be carried over or remain adsorbed to the egg to prevent the inhibition by destroying the dermal secretion. This explanation might suffice for the experiments with protease-treated eggs. However, prior to exposure to dermal secretion the trypsin-treated eggs were exposed to an amount of trypsin inhibitor (ovomucoid, four experiments with *Arbacia* eggs; soybean inhibitor, one experiment with *Lytechinus* eggs) sufficient to inhibit all trypsin activity. Furthermore, in one experiment the proteolytic enzyme treatment was applied after exposure of the egg to dermal secretion. This post-treatment with enzyme was as effective as pretreatment in rendering dermal secretion-treated eggs fertilizable. Furthermore, in preliminary experiments the fertilization inhibiting action of dermal secretion was not appreciably affected by long exposure to trypsin. Clearly, then, trypsin does not render eggs insensitive to dermal secretion by inactivating this inhibitor. The alternative, second possibility is that the enzyme destroys or removes some dermal secretion sensitive site or substance of the egg which is essential in normal fertilization. Such action in turn evidently exposes some alternative pathway for fertilization. This alternative pathway is reversibly inhibited by dermal secretion because enzyme-treated eggs fail to fertilize when inseminated in dermal secretion.

Relation of dermal secretion action to the block to polyspermy. The response of enzyme-treated eggs following dermal secretion treatment paralleled the enzyme-treated sea water controls not only quantitatively but also qualitatively. As is well known, eggs treated with proteolytic enzymes undergo polyspermic development at sperm concentrations considerably lower than that required to obtain polyspermy in normal eggs. This susceptibility to polyspermy was not confined to enzyme-treated control eggs. Thus, in the enzyme-pretreated group of experiment 2, Table III, dermal secretion-treated eggs yielded at least as high an incidence of abnormal (polyspermic) cleavage as the enzyme-treated sea water controls. This was true at all sperm dilutions.

These observations suggest that the site of action of dermal secretion may be associated in some way with the block to polyspermy. Accordingly it seemed of interest to test eggs pretreated with another polyspermy inducing agent for sensitivity to the fertilization inhibiting action of dermal secretion. Nicotine has long been known to be a very effective polyspermy inducing agent (see Rothschild, 1953; Hagström and Allen, 1956, for literature) and was therefore selected as the polyspermy inducing agent. Nicotine diluted to 2×10^{-3} in sea water proved to be optimal for inducing polyspermy in *Arbacia* eggs. However, in two experiments such treatment did not lower the sensitivity of *Arbacia* eggs to the fertilization inhibiting action of dermal secretion. When tested quantitatively over a wide range of sperm concentrations, the nicotine-pretreated eggs showed the same degree of fertilization inhibition as control eggs after exposure to dermal secretion. Apparently, then, the sensitivity of eggs to the dermal secretion is not invariably associated with the block to polyspermy.

Relation of the fertilization inhibiting action of dermal secretion to fertilizin. As suggested above, the fertilization inhibiting action of dermal secretion and its reversal by proteolytic enzymes is most readily explained by assuming a combination of the inhibitor with an essential egg substance. Such combination blocks the essential substance and prevents it from entering into reactions involved in the initial stages of fertilization. The substance that immediately suggests itself is fertilizin, and especially so since dermal secretion prevents combination of fertilizin with sperm by inactivating the sperm receptor sites of the fertilizin molecule (Metz, 1959a). If the dermal secretion inhibits fertilization by an essentially irreversible combination of the inhibitor with fertilizin at or near the egg surface, then the fertilization inhibiting action of the dermal secretion should be destroyed by addition of an excess of fertilizin.

TABLE IV
Effect of fertilizin on fertilization inhibiting action of dermal secretion

D.S. dilution	D.S. diluted in fertilizin		D.S. diluted in S.W.		S.W. + fertilizin		S.W. alone	
	Cleavage	Sperm aggluti- nation	Cleavage	Sperm aggluti- nation	Cleavage	Sperm aggluti- nation	Cleavage	Sperm aggluti- nation
1/2	0% (200 ⁺)	—	0% (200 ⁺)	—	99% (136)	++++	100% (200 ⁺)	+
1/10	0% (200 ⁺)	+++ ++++	0% (200 ⁺)	±				
1/50	13% (159)	++++	6% (140)	+				
1/250	97% (119)	++++	86% (131)	+				

Aliquots of a dermal secretion preparation were diluted serially in sea water and in fertilizin as indicated, two drops of unfertilized *Arbacia* eggs were then added to 0.8-ml. samples of each D.S. dilution, and finally 5 ml. 0.0003% *Arbacia* sperm were added to each sample. % = per cent cleavage; values in parenthesis are the number of eggs counted. To test for fertilizin excess in the mixtures 2-drop samples of the supernatant from each mixture were withdrawn after insemination of the eggs. These were mixed with 2 drops 1–2% sperm and the agglutinating action recorded on a — to ++++ scale in the table. The sperm agglutination titer of the original fertilizin solution was >729.

To test for such inactivation of the fertilization inhibiting action of the dermal secretion by fertilizin, three experiments using *Arbacia* eggs were performed. In these experiments dermal secretion was diluted serially in fertilizin, constant amounts of eggs and sperm were added to the mixtures and the percentage of cleavage determined and compared with appropriate controls. The mixtures were also tested for presence of excess fertilizin by examining for sperm agglutinating action. One experiment, typical of the three, is given in Table IV. As seen in the controls the concentration of sperm used was sufficient to fertilize 99% of the eggs. However, it was necessary to dilute the dermal secretion to 1/250 to approach this value. Furthermore, the dermal secretion inhibited fertilization to the same dilutions (1/50 to 1/250) whether diluted in fertilizin or sea water. Indeed, at dilution 1/50, five times the dilution required to achieve fertilizin excess, only 13% of the eggs fertilized. This parallel loss of fertilization inhibiting action

when diluted in fertilizin and sea water, combined with the demonstration of a fertilizin excess at 1/10 dilution, clearly shows that the fertilization inhibiting action of dermal secretion is not impaired by fertilizin. Evidently, then, fertilizin does not neutralize the fertilization inhibiting action of dermal secretion. Therefore, it seems unlikely that dermal secretion inhibits fertilization by combination with fertilizin.

It should be noted that fertilizin treatment alone did not have a marked effect on fertilization in these experiments. This is in keeping with previous observations on *Arbacia* (Tyler and Metz, 1955). However, fertilizin treatment does have a marked effect in some other species (Tyler, 1941).

DISCUSSION

The experiments described here show that the dermal secretion from *Arbacia* inhibits fertilization by action on the egg, not the sperm. The agent presumably blocks an essential reaction or reactions in the initial stages of fertilization. One such response of the egg that is evidently blocked by the dermal secretion is the formation of the fertilization membrane. In fact, treatment with dermal secretion a few seconds after fertilization arrests membrane formation. Such eggs have membrane elevated over only a part of the egg surface. It will be of interest to determine if other cortical phenomena of fertilization, such as cortical granule discharge (Motomura, 1941; Runnström, 1949; Endo, 1952) and the changes in optical properties observed in dark field illumination, are also arrested by the dermal secretion. These observations should give some indication of whether the agent inhibits the propagation of the cortical responses of the egg or interferes only with the process of membrane elevation (see Metz, 1957, for interrelations of cortical phenomena).

Unfortunately, the relationship of the blebs formed by inseminated oocytes to stages of fertilization of normal eggs is somewhat obscure. Therefore, no detailed interpretation of such inhibition in terms of fertilization is warranted. However, the blebs suggest that some of the initial stages of fertilization have taken place. Accordingly, inhibition of bleb formation by the dermal secretion indicates that this agent can inhibit initial steps in fertilization. It is not unlikely that the dermal secretion inhibits essential steps in the interaction of the sperm and the eggs, as well as arresting manifestations of the propagated responses of the egg.

The fertilization inhibiting action of the dermal secretion presumably results from interaction of the inhibiting agent with some essential substance or substances of the egg. Such interaction evidently results in destruction or blockage of the essential substance. This in turn reduces the number of receptor sites on the egg and the probability of a successful sperm-egg contact. This view is consistent with the observation that inhibitor-treated eggs can sometimes be fertilized if inseminated with high sperm concentrations.

Cases of cleavage delay may also be explained in formal fashion as a reduction of the probability of a successful sperm-egg collision. Thus eggs with a reduced number of receptor sites should have a lower "fertilization rate" (*e.g.* Rothschild, 1956; Hagström and Hagström, 1954) than control eggs because of the increased time required for the less probable event. Another possibility is that cleavage delay is a manifestation of a reversal of inhibition such that washed dermal se-

cretion-treated eggs undergo a slow reversal of inhibition. It should be noted, however, that other factors such as delay in the propagated response of the egg have not been eliminated as explanations for delayed cleavage of dermal secretion-treated eggs.

The fertilization inhibiting interaction between the dermal secretion and the essential egg substance could result in enzymatic destruction of the latter. It appears more likely, however, that the inhibitor acts by relatively undissociable combination with an egg substance. This view is supported by the observation that washing sometimes results in at least partial restoration of fertilizability to dermal secretion-treated eggs. It is also consistent with the observed reversal of inhibition by proteolytic enzymes.

The identity of the egg substance or substances with which the dermal secretion combines to inhibit fertilization remains obscure. This material is evidently not the sperm isoagglutinin, fertilizin. Several lines of evidence support this view. Fertilizin constitutes the jelly layer surrounding the egg. Eggs from which this jelly has been removed by acid sea water treatment are dermal secretion sensitive. Evidently, then, fertilization inhibition is not mediated by action of the dermal secretion on the jelly. However, this experiment does not rule out the possibility of action on an acid-resistant layer of fertilizin bound to the egg surface (*e.g.* Tyler, 1941). Further "dissection" of the egg surface was achieved by treating eggs with proteolytic enzymes. Following such treatment the eggs were relatively insensitive to the fertilization inhibiting action of dermal secretion. Evidently the enzyme digests or otherwise eliminates the primary egg substance with which the dermal secretion combines. However, there is some question if even the relatively drastic action of proteolytic enzymes removes all the fertilizin from the egg surface (Tyler and Metz, 1955) in a reasonable time. As a final test for a role of fertilizin in the inhibition of fertilization by dermal secretion, fertilizin and dermal secretion were mixed and the mixtures were assayed for fertilization inhibiting action. The fertilizin had no effect on the inhibiting action of dermal secretion. This was true even when the fertilizin was present in large excess as measured by sperm agglutinating action.

In view of these results it appears that the dermal secretion does not inhibit fertilization by an action on fertilizin. This is of particular importance because previous studies (Metz, 1959a) have shown that dermal secretion of *Arbacia* destroys the sperm combining sites of the fertilizin molecule.

Apart from consideration of the nature of the egg substance involved in the inhibition, the reversal of inhibition by either "pre- or post-" treatment of eggs with proteolytic enzymes has interesting implications for other aspects of fertilization. According to the interpretation given above dermal secretion inhibits fertilization by combination with a substance that is essential for fertilization of the normal egg. Pretreatment of the egg with proteolytic enzymes removes this essential egg substance and renders the egg relatively insensitive to the inhibitor. Evidently coincident with the removal of the essential egg substance by enzyme, an alternative pathway(s) to fertilization is exposed. Whether this pathway resides in the sperm-egg attachment mechanism, the activation initiating mechanism or the propagative and cortical response mechanism remains to be established. However, it should be noted that the "alternative pathway" exposed by proteolytic

enzyme treatment is associated with a loss of the block to polyspermy and a reduction in fertilization specificity (Bohus Jensen, 1953; Hultin, 1948a and 1948b; Tyler and Metz, 1955) and is itself reversibly inhibited by dermal secretion. This last conclusion follows from the fact that enzyme-treated eggs must be washed free of dermal secretion before they will fertilize. Whatever the site of action of the proteolytic enzymes the action does not result in drastic visible effects on the unfertilized egg, for sections of trypsin-treated and control eggs are indistinguishable when seen with electron optics (Parpart, personal communication).

This investigation suggests the use of fertilization inhibiting agents as markers or labels for particular sites or substances involved in fertilization. Extension of the investigation to include detailed examination of other fertilization inhibitors should prove interesting for these may act at other sites. Indeed, the studies of Branham and Metz (1959) indicate that the inhibition of fertilizin agglutination of sperm by extracts from *Fucus* and by dermal secretion results from two quite different mechanisms. It will not be surprising if these two agents are found to inhibit fertilization by action at different sites in the complex of reactions that result in fertilization.

SUMMARY

1. Sperm washed from *Arbacia* dermal secretion fertilize eggs as readily as sperm washed from sea water.

2. Eggs washed from *Arbacia* dermal secretion do not fertilize as readily as controls. At best such eggs require high sperm concentrations to achieve fertilization. In some experiments the treated eggs show a marked cleavage delay. It is concluded that the dermal secretion inhibits fertilization by action on the egg, not on the sperm.

3. Addition of dermal secretion to eggs several minutes after insemination has no effect on the development of the eggs. Similar treatment a few seconds after insemination results in arrest of fertilization membrane elevation and sperm penetration. The dermal secretion also inhibits the formation of blebs in inseminated oocytes. It is concluded that dermal secretion inhibits by blocking an initial stage(s) in fertilization.

4. Jellyless (acid-treated) eggs fail to fertilize after treatment with dermal secretion. They have undiminished sensitivity to the inhibiting action of the dermal secretion.

5. Proteolytic enzyme-treated eggs are relatively insensitive to the inhibitor. They fertilize as readily as controls after treatment with dermal secretion, provided they are washed free of this inhibitor. They fail to fertilize if inseminated in dermal secretion.

6. The dermal secretion has undiminished fertilization inhibiting action in the presence of an excess of fertilizin.

7. The experiments suggest that the dermal secretion inhibits fertilization by combining with some egg substance that is essential for fertilization of the normal egg. The egg substance is apparently not the sperm isoagglutinin, fertilizin. Treatment of the egg with proteolytic enzymes eliminates the essential egg substance and simultaneously exposes an alternative pathway to fertilization. The latter is reversibly inhibited by dermal secretion.

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PROTEIN CHANGES IN DEVELOPMENT^{1, 2}

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Biologists have long been in agreement with the thesis that morphological differentiation is either preceded by, or accompanied simultaneously by, an underlying chemical differentiation. In the testing of this hypothesis, considerable attention has been focused on the qualitative and quantitative changes occurring in the proteins of developing embryos.

The usual biochemical techniques have been employed to describe and to quantitate these changes. One should mention the solubility studies of sea urchin embryo proteins by Mirsky (1936), the electrophoresis of sea urchin embryo proteins by Monroy (1950), and the electrophoresis of amphibian embryo proteins by Flickinger and Nace (1952). Recently the serological technique has been used quite extensively; the precipitin test by Cooper (1948), Ebert (1951), Harding *et al.* (1955); the Oudin technique by Spar (1953) and by Cooper (1948).

In addition to these *in vitro* studies a number of *in vivo* studies have been carried out. Ebert (1953, 1955) and his co-workers have studied the effects of specific antisera on living chick embryos, and Tyler and Brookbank (1956) have studied the cytotoxic effects of specific antisera on sea urchin eggs. We have carried out similar studies on dissociated sponge cells (Spiegel, 1955). The results of these investigations have been extensively summarized by Nace (1955) and by Tyler (1957).

Briefly, the results obtained through the use of the serological techniques on amphibians and sea urchins are as follows. In the sea urchin, it has been demonstrated by Perlmann and Gustafson (1948) and Perlmann (1953) that new antigens appear during development. These new antigens were first detected in the gastrula stage. Harding *et al.* (1954, 1955), working with lethal hybrid sea urchin embryos, were able to detect paternal antigens at the blastula stage.

In the frog, it seems fairly well established from the work of Cooper (1946, 1948, 1950) and of Spar (1953) that new antigens are found in the blastula and gastrula stages. Ten Cate and Van Doorenmaalen (1950) and Flickinger *et al.* (1955) have shown that there is good correlation between the time of appearance of lens antigen and of the lens itself. Flickinger and Nace (1952) have detected new antigens in the tail-bud stage.

Clayton (1953) has further demonstrated that between the blastula and gastrula stage, before neurulation, and between neurulation and the formation of the tail-bud, a synthesis of antigenic material occurs. Ectoderm and archenteron roof contain fractions specific to themselves as well as having common antigens.

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² Part of this work was carried out in the Department of Biology, Colby College, Waterville, Maine.

A word of caution seems warranted when considering these results. It should be mentioned that Tyler (1957) has pointed out that in investigations of this kind (in which antisera produced against saline extracts—after suitable absorptions—have been used to detect changes in antigens) there is the uncertainty as to whether or not a particular antigenic structure remains associated with a saline-soluble constituent and as to whether or not it is available for reaction in specific absorption procedures. The appearance of new antigens or the loss of old antigens may represent a change in solubility, rather than the synthesis or destruction of an antigen. The above evidence, therefore, which has been represented as a synthesis of new material may rather reflect changes in solubility of pre-existing substances. This point will be more fully considered in the discussion of the results presented in this paper.

The study to be reported here involved the use of the technique of zone electrophoresis to follow the changes occurring in the proteins of developing embryos and to compare these changes with adult organ proteins.

MATERIALS AND METHODS

I. *Preparation of extracts*

A. *Developmental stages.* Embryos of the frog, *Rana pipiens*, from the unfertilized egg through stage 21 (Shumway, 1940) were used. Eggs were obtained by pituitary injection of large adult females and fertilized by the usual method of stripping directly into a suspension of macerated testes in 10% Holtfreter's solution, pH 7.8. The embryos were then washed three times with sterile 10% Holtfreter's solution, pH 7.8, and incubated at 19–20° C. until the desired stage was attained. Extracts were prepared from embryo populations in which at least 95% of the embryos were of the same stage and developing normally. Regardless of the stage at which the extract was made, 100–200 embryos of each population were always carried through stage 22 and if subsequent development of these controls showed more than 5% of the embryos to be abnormal, the extract was discarded. For the unfertilized egg extracts, 100–200 eggs were fertilized and incubated as described above. If more than 5% of the embryos were abnormal, the extract was discarded.

The jelly of individuals of the desired stage was then removed by the papain-thioglycolate method of Spiegel (1951). After washing five times with sterile 10% Holtfreter's solution, pH 7.8, 500–1000 jelly-free embryos or eggs were allowed to settle out, under gravity, in a 50-ml. cellulose nitrate centrifuge tube. Excess supernatant fluid was removed by aspiration, leaving only the small amount of solution that was trapped in the interstices between individuals. The loosely packed embryos were frozen-thawed, homogenized in a glass homogenizer, and centrifuged at 23,000 *g* for 10 minutes. The clear supernatant fluid minus the fat layer was removed by aspiration and stored at –20° C. until used. All developmental stages were treated in identical fashion. The temperature throughout the extraction procedure was 4° C.

B. *Adult organs.* The following organs were dissected from adult frogs of both sexes: brain, gastrocnemius muscle, and small intestine. These organs were then extracted by the identical method used for developmental stages. Adult blood

was obtained by cardiac puncture from adult male and female frogs, diluted with an equal amount of 10% Holtfreter's solution, pH 7.8, centrifuged at 23,000 *g* for 10 minutes, the supernatant fluid, strongly colored by hemoglobin, removed by aspiration, and stored at -20°C . until used.

II. *Electrophoresis*

Before electrophoresis, the extracts were thawed and dialyzed in the cold vs. three changes of the electrophoretic buffer, Veronal, pH 8.6, $\mu = 0.05$, for 24 hours. The dialysates were centrifuged at 23,000 *g* for 10 minutes and 0.02 ml. of supernatant fluid was applied to filter paper strips (Spinco Part No. 300-028). The 8-strip Spinco Durrum-type electrophoresis cell and the Spinco Model R regulated power supply were used. Electrophoresis was carried out at room temperature using 25 ma constant current for 6 hours. Under these conditions rather remarkable reproducibility was obtained. Each stage or adult organ extract was prepared a minimum of five times and each preparation subjected to at least three separate runs, making a total of at least 15 electrophoretic runs per type of extract.

III. *Staining and scanning of paper strips*

At the completion of the run, the papers were dried at 115°C . for 30 minutes and stained by the rapid brom phenol blue procedure for proteins described by Durrum (1958). The strips were rinsed in methanol for 6 minutes, stained for 30 minutes in 0.1% brom phenol blue in methanol, followed by three rinses in 5% acetic acid of 6 minutes duration per rinse. They were then blotted, dried at 115°C . for 15 minutes, exposed to concentrated ammonium hydroxide vapor for 15 minutes, and scanned with the Photovolt Densitometer Model 525 (using a 505-millimicron broad band filter) coupled to the Varicord Variable-Response Recorder. For glycoprotein identification, the periodic acid-fuchsin sulfite method of K6iwi and Gronwall (1952), as described by Durrum (1958), was used. The strips were scanned as described above.

RESULTS

A. *Developmental stage extracts*

The brom phenol blue protein pattern obtained with unfertilized egg extracts is shown in Figure 1. Seven cation-bands and two anion-bands were noted. We chose to use the term bands rather than proteins since the absence of additional data such as electrophoresis at pH's other than 8.6, salt fractionation, etc., did not allow us to conclude that each band represented a single protein. Indeed, careful examination of the densitometer records often revealed that a major band was composed of two or more small peaks. For example, the cation-band labelled B in Figure 1 was actually composed of three smaller peaks, readily reproducible from run to run. In addition to these stained bands there was an additional band, labelled β , which was not stained by brom phenol blue but which charred as a result of drying at 115°C . for 30 minutes and appeared as a yellow-brown band in the unstained strip. It was the only band which fluoresced under ultraviolet illumination. It did not correspond to any of the brom phenol blue-stained bands

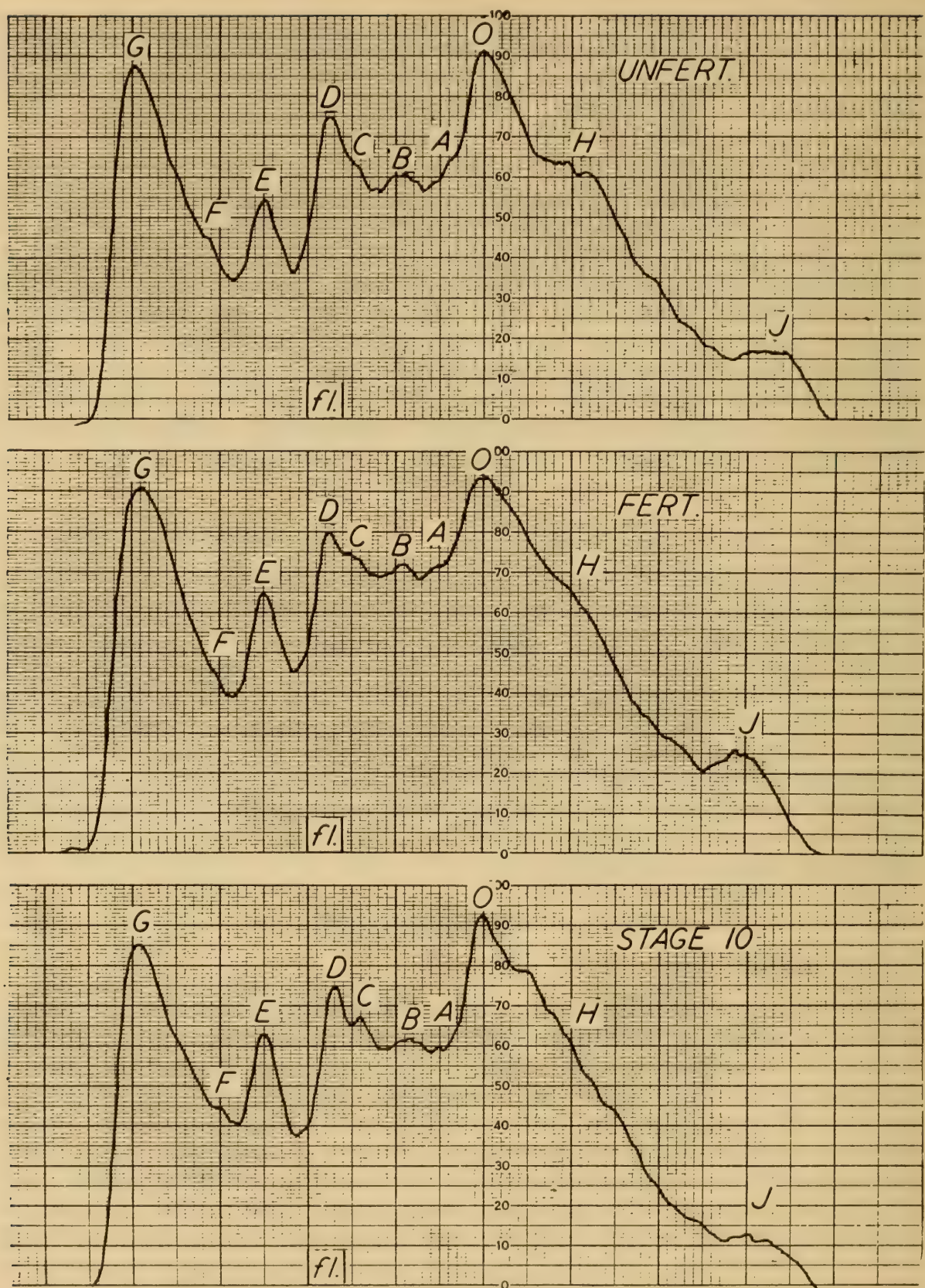


FIGURE 1. Densitometer tracings of brom phenol blue-stained paper strips of developmental stage extracts after electrophoresis. Ordinate labelled 0 represents point of application. Values 0-100 on the 0 ordinate represent the relative density of brom phenol blue stain. To the right of the 0 ordinate are the anions; to the left are the cations. Abscissa units represent relative distance away from the point of application.

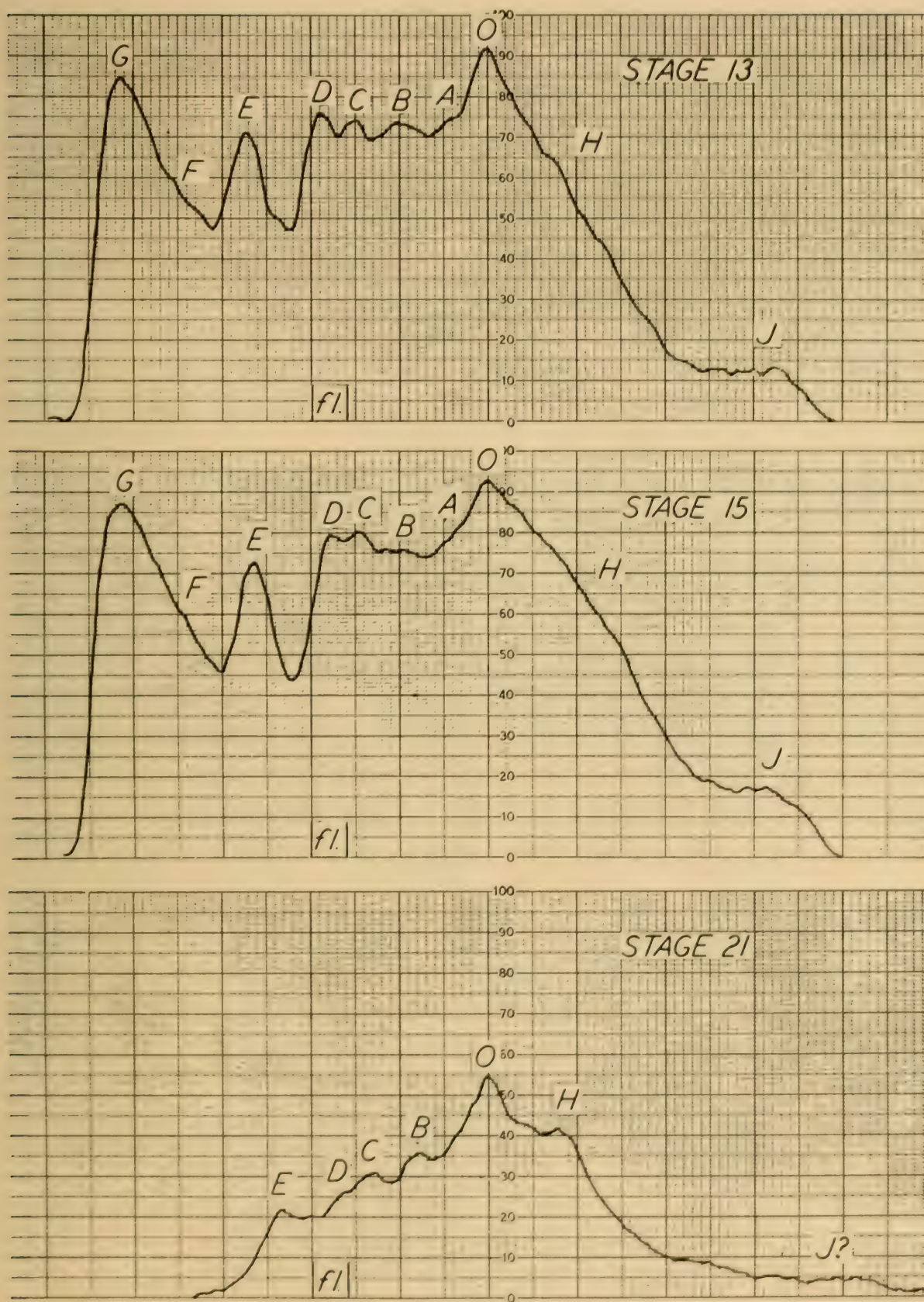


FIGURE 2. Densitometer tracings of brom phenol blue-stained paper strips of developmental stage extracts after electrophoresis. Symbols as in Figure 1.

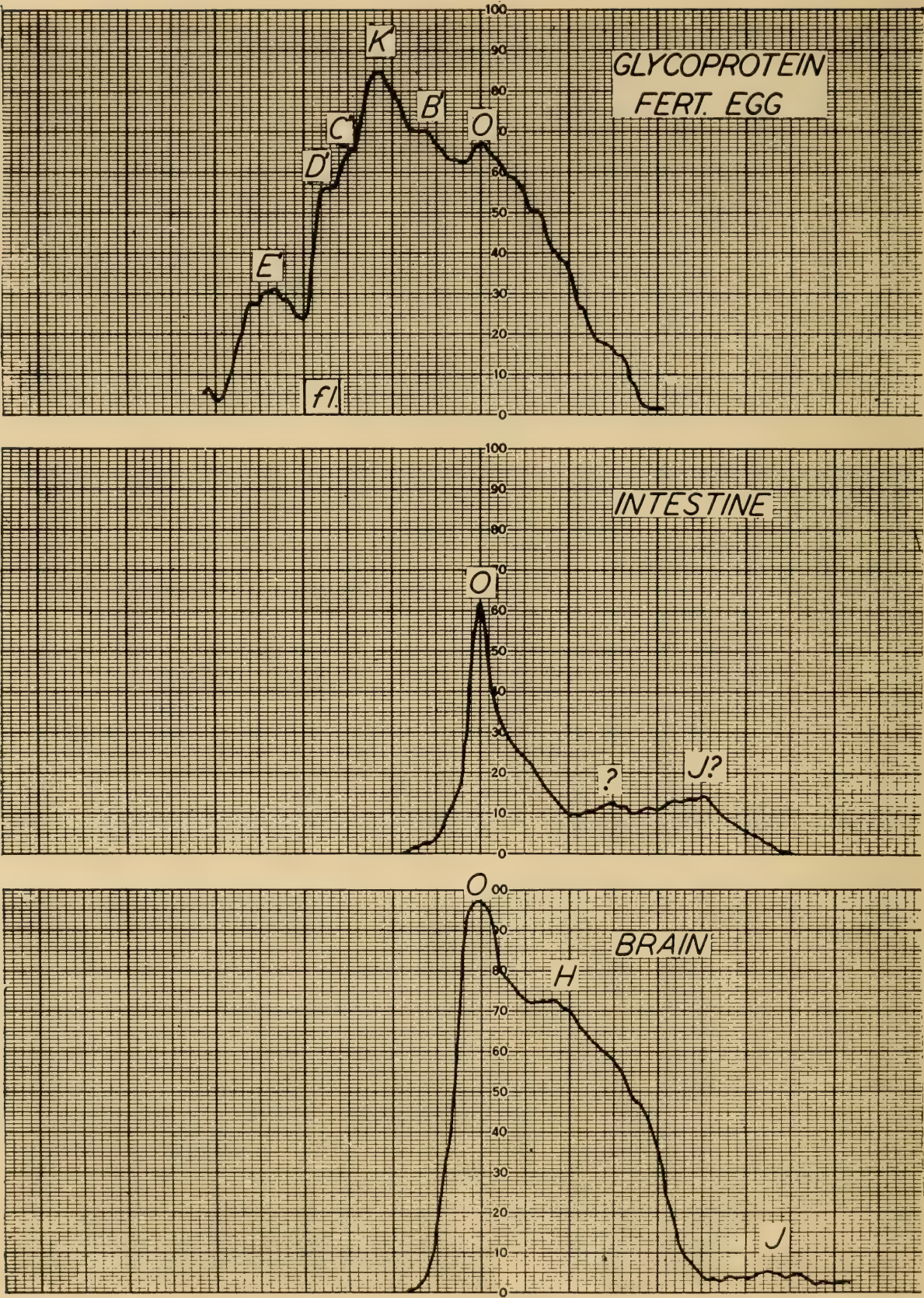


FIGURE 3. Upper: Densitometer tracings of periodic acid-fuchsin sulfite-stained paper strips of developmental stage extracts after electrophoresis. Middle and bottom: Densitometer tracings of brom phenol blue-stained paper strips of adult organ extracts after electrophoresis. Symbols as in Figure 1.

and its limits were demarcated in the figure by the two lines on either side of the label *f*l in the figures.

The patterns obtained with fertilized egg extracts and stage 10 embryo extracts are shown in Figure 1. It was readily noted that following fertilization there was a decrease in the concentration of the H band which was perhaps comparable to the "Mirsky protein" found in the sea urchin egg. There was also a quite noticeable progressive increase during development in the concentration of the cation-band C. All other bands remained essentially constant in concentration through stage 15 (Figs. 1 and 2).

By stage 21, bands A, F and G were no longer detected (Fig. 2). The presence of band J was questionable and at best was in very low concentration and migrated as a broad band rather than as a sharply defined entity. The over-all protein concentration, under the conditions of extraction and electrophoresis employed, was markedly reduced. Band H, however, increased in relative concentration and by stage 21 approximated the relative concentration found in the unfertilized egg.

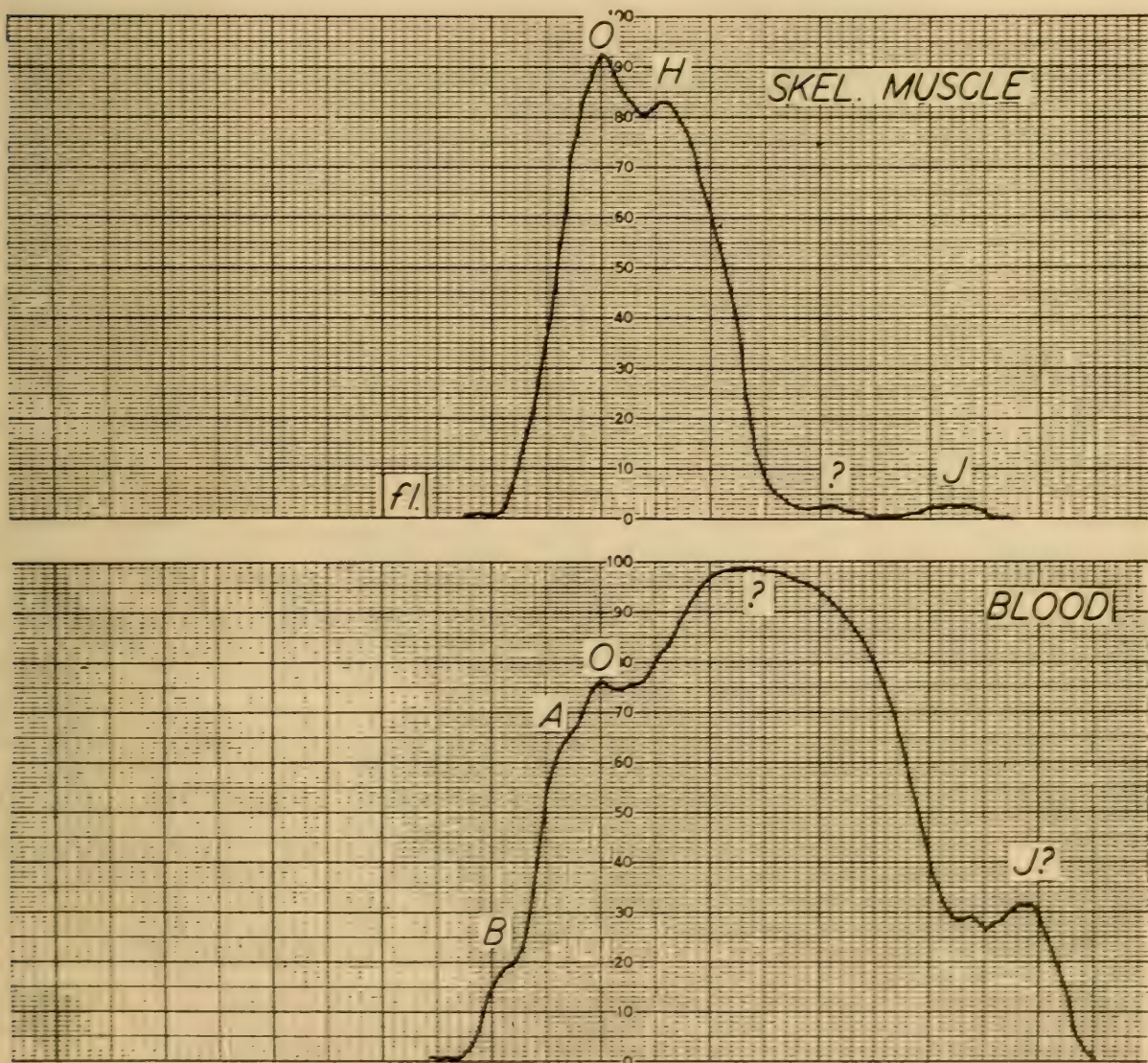


FIGURE 4. Densitometer tracings of brom phenol blue-stained paper strips of adult organ extracts after electrophoresis. Symbols as in Figure 1.

Staining with the periodic acid-fuchsin sulfite method for glycoproteins revealed the presence of 5 stained bands, all migrating as cations (Fig. 3) in the fertilized egg extract (two hours after fertilization). One of these bands, K', did not correspond to any of the brom phenol blue-stained bands. The remaining bands, B', C', D' and E', corresponded in position to the B, C, D and E bands, respectively, of the brom phenol blue-stained strips.

The periodic acid-fuchsin sulfite pattern of the unfertilized egg extract was identical with the above results. Through stage 15, however, there was a progressive decrease in the stainability or concentration of these bands and by stage 21, the presence of carbohydrate was no longer detected. Reasons for labelling these bands with primes are considered in the discussion section.

B. Adult organ extracts

Patterns obtained with extracts of adult organs are demonstrated in Figures 3 and 4. These patterns revealed that relatively few of the ion-bands present in

TABLE I
Brom phenol blue-stained proteins of adult organ extracts present in unfertilized egg extracts

Adult organ extract	Band				
	<i>f</i>	B	A	H	J
Intestine	—	—	—	—	±
Brain	—	—	—	+	+
Skeletal muscle	+	—	—	+	+
Blood	—	+	+	—	±

+ Indicates presence of band.

— Indicates absence of band.

developmental stages were detected in adult organ extracts. The majority of the cation-bands were not detected; in intestine and brain extracts, none were detected; in skeletal muscle only the *f* band was detected; in the blood extract only the A and B bands were present. All of the anion-bands were detected, although not all were present in each adult organ extract.

Bands labelled with a question mark in the figures do not necessarily represent new or different proteins but rather represent pre-existing proteins which were unmasked by the changes in concentration of substances having almost identical mobilities. In addition, the anion-band labelled with a question mark present in the blood extract probably represents the serum proteins masked by hemoglobin. In the unstained strip the band was red in color.

The results obtained with adult organ extracts are summarized in Table I.

DISCUSSION

It is apparent from the results summarized in Table I that although the majority of the proteins extracted from adult organs are present in the unfertilized egg, the

protein patterns of adult organ extracts do exhibit differences. There are some components present in more than a single organ but others are apparently confined to a single organ. For example, bands H and J are present in both skeletal muscle and in brain extracts; the *f* band, on the other hand, is present only in the skeletal muscle extract.

These patterns suggest that all, or most, of the proteins found in adult organs are first found in the unfertilized egg. As development proceeds some of the cells lose their ability to manufacture one or more particular proteins. Correlated with this loss of ability to synthesize a particular protein is a corresponding morphological differentiation. Differentiation, therefore, could involve a loss of the ability to synthesize a protein rather than the synthesis of a new protein. The work of Ebert (1953, 1955) on the distribution of cardiac myosin in the chick embryo is in support of this hypothesis. Ebert demonstrated that early in development cardiac myosin is rather uniformly distributed throughout the blastoderm. As development proceeds, the ability to synthesize cardiac myosin is eventually restricted to those cells located in the two heart-forming regions.

An alternative to the above explanation is equally probable. The differences observed in the protein patterns of adult organs and those of the embryo may reflect changes in the solubility of the proteins of the unfertilized egg or of adult organs rather than the loss or gain of any synthetic capacity. As a result of this line of reasoning, one could assume that the same proteins are present in the adult brain as in the unfertilized egg, but some of these proteins are insoluble, perhaps under the conditions of extraction, in the adult organ. At present, the more probable hypothesis cannot be determined. It is likely that both processes are involved in differentiation. The solubility and location of the proteins detected in this investigation are unknown. Whether we are dealing with the soluble proteins of the cytoplasm or those proteins soluble in the small amount of 10% Holtfreter's solution (and ultimately in the electrophoretic buffer) used for extraction is not readily apparent. Further studies are necessary to determine location, solubilities, etc. of these proteins. Why the present work does not show the appearance of "new" proteins (proteins insoluble in the unfertilized egg but soluble at later stages)—if the latter hypothesis is correct—cannot be answered at the present time.

It must be emphasized that the identification of proteins in this study has been made solely on the basis of mobility in an electric field. It is possible that new and different proteins from those of the unfertilized egg are being synthesized throughout development. These new proteins could have the same or closely matching mobilities as those of the early embryo, would therefore not be detected as new proteins, and could perhaps give the impression of a static population of protein types which does not exist. Further characterization of these proteins by other biochemical techniques will resolve this question. Of interest is the observation that, under these conditions, the majority of the embryonic proteins are cations; those of the adult are primarily anions. It is possible that those cation-bands which are lost during development represent the yolk proteins and are utilized as an energy source.

If the over-all hypothesis be true, that differentiation can be brought about by, or is associated with, the loss of the ability to synthesize a pre-existing protein

and/or changes in solubilities of pre-existing proteins, one must consider the ever-increasing body of literature apparently demonstrating the presence of new antigens in development. All of these papers, to our knowledge, have dealt solely with saline-soluble antigens. The appearance of a new antigen in development may rather represent a change in solubility rather than a synthesis of new antigenic material. For example, it is entirely possible that an antigen present in the blastula stage is saline-insoluble but in the gastrula stage is soluble in saline. Such an antigen, after the usual absorptions, etc. have been carried out, would then be described as a new antigen arising during gastrulation rather than as a change in solubility of a pre-existing substance. The demonstration by Markert and Møller (1959) of multiple forms of enzymes—the isozymes—which are tissue, ontogenetic, and species-specific can also be explained, rather simply, on the basis of changes in solubility.

The recent paper by Solomon (1959) offers a striking demonstration of changes in location, and therefore of solubility, of a particular enzyme during development. It was shown that the mitochondrial glutamic dehydrogenase of the chick embryo liver increased in activity after the twelfth day of incubation and this rise was followed by a sudden drop of glutamic dehydrogenase activity in the supernatant fluid (0.25 *M* sucrose) after 15 days of incubation. Supernatant fluid glutamic dehydrogenase activity is four times that of mitochondria at 7 days' incubation; 6 times at 12 days' incubation; $\frac{1}{3}$ times at 20 days' incubation. The thesis that changes in the intra-cellular distribution of enzymes may occur during differentiation has been put forth by Krahl (1950), and Gustafson (1954).

The work of Schechtman (1955) and Nace (1953) has further demonstrated that the serum proteins of the chick are present in the unincubated egg and are probably transferred from the maternal circulation to the egg.

The results obtained using the periodic acid-fuchsin sulfite staining technique for glycoproteins remain to be considered (Fig. 3). The band K' does not correspond to any of the brom phenol blue-stained bands and is probably a polysaccharide. The fact that the remaining four cation-bands, B', C', D' and E', correspond in position to the B, C, D and E bands, respectively of the brom phenol blue-stained strips, coupled with the observation that the periodic acid-fuchsin sulfite-stained bands were detected in early stages but not in stage 21 extracts, has led to the following interpretation.

It is possible that these bands represent a polysaccharide migrating at the same rate as proteins composing the band, or they may represent a glycoprotein. By stage 21, synthesis of these polysaccharides or glycoproteins has ceased or their solubility has changed. It would therefore be justifiable to label these bands as distinct from other proteins present in the band. It is also possible that the periodic acid-fuchsin sulfite technique is less sensitive than the brom phenol blue technique. Due to the decrease in protein concentration of the stage 21 extracts (Fig. 2) a positive brom phenol blue test but negative polysaccharide test results. Which of these interpretations is correct remains to be determined. The labelling of these bands with prime letters indicates the possibility of their being discrete entities.

It is realized that the present paper by no means offers conclusive proof, but merely suggests that differentiation may, in part, involve the loss of ability to synthesize a particular protein and/or changes in the solubility of pre-existing

proteins. The intra-cellular locations, concentrations, tissue distributions, and further biochemical characterizations of these proteins are being carried out in this laboratory at the present time.

SUMMARY

1. Extracts of developmental stages and of four adult organs of the frog, *Rana pipiens*, were examined by the technique of paper electrophoresis.
2. Seven protein cation-bands and two protein anion-bands stained with brom phenol blue were detected in extracts of developmental stages from the unfertilized egg through stage 15. By stage 21, two of the cation-bands were no longer detected.
3. Examination of the adult organ extracts revealed organ-specific proteins in addition to proteins common to all organs tested.
4. The results were interpreted in terms of the hypothesis that differentiation involves, in part, the loss of ability to synthesize a particular protein(s) and/or changes in the solubility of pre-existing proteins.

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TISSUE SPECIFICITY IN EMBRYONIC AND ADULT CYMATOGASTER AGGREGATA STUDIED BY SCALE TRANSPLANTATION

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The method of scale transplantation first used by Mori (1931) has proved to be of great value in studies on tissue specificity in fishes. Goodrich and Nichols (1933) and Hildemann (1956, 1957a, 1957b) have shown that, whereas autografts are always successful, homografts are invariably rejected by the host. These studies have established that the immune responses to homografts so profusely studied in mammals and birds are also observable in the goldfish (*Carassius auratus*).

The purpose of this investigation was to extend our knowledge of homograft reactions in fishes. Answers were sought to the following questions: (1) To what extent are adults of *Cymatogaster aggregata* (Gibbons) immunologically reactive toward scale homografts? (2) Are adults of this species capable of being sensitized to scale homografts (*i.e.*, will secondary, tertiary, etc., grafts be more rapidly rejected than the primary graft)? (3) Does the reactivity of embryonic and immature *C. aggregata* toward scale homografts differ from that of the adults? (4) Assuming affirmative answers to the above questions, can a sensitized, pregnant female transfer this homograft sensitivity to intra-ovarian embryos?

MATERIALS AND METHODS

Experimental animals

C. aggregata is a marine embiotocid fish which is distributed over the west coast of North America from southern Alaska through Baja California (Tarp, 1952). Like all embiotocids this species is viviparous, the young spending about five months in the hollow ovary and increasing in size from 0.25 mm. (fertilized egg) to about 42 mm. (newborn) in standard length (Eigenmann, 1889; Triplett, unpublished data). Animals used in this investigation were collected by angling from Goleta pier, Goleta, California.

Methods

The method of grafting was essentially similar to the one devised by Hildemann (1957b). Adult fish were anesthetized in tricaine methane sulfonate (MS 222), 1/5000 dissolved in one per cent sodium chloride (MS 222 forms a white precipitate in sea water). Survival was greatest when the anesthetic was maintained at about 10° C. Grafting was done simply by removing a scale from the ventral surface of the host and replacing it with a scale from the dorsal surface of the

same fish or another fish. The transplant is easily distinguished since the scales of the dorsal surface of the body are heavily pigmented in contrast to those of the ventral surface which contain few or no melanophores. Grafted adults were maintained in aquaria provided with running sea water. They were fed *ad libitum* on pieces of mussel (*Mytilus californianus*).

Embryos were obtained by caesarian section using aseptic technique. They were then transferred to fingerbowls containing a modification (Triplett, unpublished data) of sterile White's medium (1954) and kept there until time was available for grafting. The embryos were anesthetized in 1/10,000 MS 222 dissolved in the aforementioned solution. An incision was then made in the flank skin with iridectomy scissors. Into this was placed either a homograft consisting of a sterile, adult, trimmed scale, or an autograft consisting of a piece of pigmented skin. The scales of embryos, when present, are too small and lightly pigmented to be used as autografts. Adult scales were sterilized by immersion for one minute in 0.004 *M* mercuric chloride dissolved in modified White's medium and washed with sterile medium before grafting. The scale remained alive and apparently healthy after such treatment. Embryos were grafted and maintained at 17° C. in a sterile culture chamber.

All host fishes, embryonic and adult, received a homograft and most fishes received an autograft. After it was established that autografts would survive and become healthy, some of the newborn in subsequent experiments were not given autografts in order to avoid this additional injury. In some cases reciprocal grafting of pairs of fishes was done. In others a single donor contributed scales to between three and eleven hosts.

The principle criterion for assaying the grafted scales was the condition of the scale melanophores. The melanophore is a single pigment cell of a radially dendritic pattern. The melanophores are usually somewhat separated from one another and readily visible under a dissecting microscope. These pigment cells contain many granules of melanin which respond to physiological changes to spread out into the cell's extremities, or to aggregate in the center of the cell. In a typical situation the melanophores of autografted scales, after an initial healing period, remained healthy and completely pigmented for an indefinite period of time. After a given time homograft melanophores began to undergo degenerative changes. The pigment aggregated as a black dot in the center of the melanophore and over a variable interval of time proceeded to fragment. The melanin granules were eventually phagocytized. In a few cases an inflammatory reaction similar to that described by Hildemann (1957b) and Goodrich and Nichols (1933) was observed. The time required for fragmentation of all the melanophores of the transplanted scale is here termed the reaction time.

RESULTS

Adults

The adults of this species, like many pelagic fishes, are difficult to maintain in a laboratory without running sea water. Therefore the temperature, of necessity, was that of the ocean at that particular time of the year. In order to measure the effect of temperature on the reactivity of animals toward homografts one series was performed between March and July (average temperature = 17° C.),

and another series was done between August and October (average temperature = 20° C.).

In these series a single donor contributed scales to either three, four or five hosts. In all, 37 hosts and eight donors survived long enough to yield data. The hosts received as many as six successive grafts from the same donor, new grafts being placed as soon as fragmentation of the melanophores of the previous graft was complete. As mentioned above all the autografts placed on hosts remained alive and healthy for the duration of the experiment (in some cases more than three months).

Both autografts and homografts underwent an initial healing phase during which time the melanophores that had been injured during the operation fragmented and were phagocytized. After the third post-operative day degenerating melanophores could no longer be found in the autografts, but progressive loss of melanophores continued in the homografts until all had disintegrated (reaction time). The reaction time for homografts is shown in Table I. Here it can be seen that the average reaction time for grafts at 17° C. is 7.0 days. The reaction time of subsequent grafts decreased progressively until at the fourth grafting it averaged 3.5 days. Any further grafting was not effective in increasing homograft sensitivity.

It can also be seen in Table I, in confirmation of Hildemann's (1957b) findings, that increased temperature of the external medium has the effect of decreasing the reaction time. It was also observed that at the higher temperature the primary homograft has a more pronounced effect in producing sensitivity to subsequent grafts. These observations are paralleled by those of Cushing (1942) who found that the ability of fishes to produce antibody against injected erythrocytes or spermatozoa is influenced by temperature in such a way that circulating antibody appears sooner at higher than at lower temperatures.

Some evidence has been gathered which indicates (as might be expected) that fishes of this species, comprising a natural population of unknown interrelationships, bear some but not all scale tissue factors (antigens?) in common. Six fish of the series discussed above, which had received between four and six successive homografts, were each given a graft from a donor that had not previously been used. The average reaction time for this series was 5.6 days. The value for a primary graft would have been 7.0 days (average), and the value for a fifth, sixth or seventh set reaction would have been 3.5 days (average). Since the realized value is in between these two, the statement that each fish bears distinctive as well as common homograft factors seems justified. Medawar (1946) has made similar observations on rabbits.

Significance of reaction time

A small series of operations was performed in an effort to determine the relationship of the reaction time (melanophore disintegration time) to the complete destruction of the living tissue of the homograft. The method, first used by Hildemann (1957b), consisted of returning homografts to the donor after variable lengths of time. Failure of the graft to re-establish itself in the original donor indicated irreparable damage or death.

In this series five pairs of adult fish were used. Each donor contributed 10

scales to its partner, and the grafted scales were returned to the donor at daily intervals. It was found in all five cases that scales returned to the host on or before the third day after the original grafting became re-established in the donor. Those returned on or after the fourth day did not survive. These data lack statistical

TABLE I

Reaction time in days for scale grafts to adult fish

Operations performed between March and July—Average water temperature 17° C.

Specimen no.	1st graft	2nd graft	3rd graft	4th graft	5th graft	6th graft
1-1	11					
2	7	7				
3	9	9	6	3	3	3
4	?	8	3	3	3	4
5	—	4				
2-1	9	11				
2	11	11				
3	11	11				
4	11	9				
5	3	10	4	4	3	
3-1	7	9				
2	6					
3	6	6	4	3		
4	6	5	6	5		
5	9	4				
4-1	11					
2	6	5				
3	11	6	3	3	4	
4	11	5	6	3	4	3
5	11	5	6	4	4	
5-1	6	7	4			
2	7	7				
3	6	7	3			
4	6	8	4			
5	6	5	5			
Average	7.0	7.2	4.5	3.5	3.5	3.3

Operations performed between August and October—Average water temperature 20° C.

1B-1	5					
2	5					
3	5	3				
4	7					
2B-1	5	3				
2	7	3				
3	5					
4	5	3				
5	7					
4B-1	7	3				
2	7	3				
3	5	3				
Average	5.8	4.0				

force but suggest that homografts are no longer viable in a suitable environment several days before the reaction time (7.0 days at this temperature). It is felt, however, that the reaction time as used in the various experiments reported here is valid since all other data indicate that it is a function of host homograft sensitivity.

Embryos

Two series of experiments were done on intra-ovarian embryos. The hosts of the first series consisted of six siblings averaging 29 mm. standard length, and the hosts of the second series consisted of 11 siblings averaging 19 mm. standard length. The hosts in each series received homografts from a single adult, the operation and postoperative care being done in the manner described above.

Since the grafts had to be examined every day, and since the medium was rich in organic materials some difficulty was experienced in maintaining asepsis. In the first series, four animals lived 16 days, one lived 20 days and one lived 24 days. In the second series eight animals lived 20 days, and three lived 30 days.

An initial healing period lasted between three and five days. During this time damaged melanophores of both autografts and homografts were phagocytized. After this time both autografts and homografts remained alive and in excellent condition as long as the host lived. Since some of the embryos lived as long as 30 days there is a strong indication that intra-ovarian animals are incapable of eliciting the homograft reaction.

Immature fishes

The animals used in this series were divided into two groups on the basis of their parturition age. There survived in the first group 11 animals grafted one to three days after birth, and in the second group seven fishes grafted 15 days after birth. Some, but not all animals received an autograft consisting of a 0.25-mm. piece of skin, and all received a scale homograft from an adult fish. Each donor contributed to between three and seven hosts. All animals in this series were cultured at approximately 17° C. in running sea water.

The autografts remained alive for the duration of the experiment. The homografts were without exception broken down in a manner similar to that described for the adult host homografts. It can be seen in the first two columns of Table II that the reaction time for homografts is approximately in inverse proportion to the age of the fish. The average reaction time for one- to three-day fishes is 13.2 days, whereas that for 15-day fishes is 9.7 days (that for adults is 7.0 days at the same temperature).

Embryos from sensitized parents

These experiments were performed in an effort to discover whether or not induced homograft sensitivity in parent fishes could be transferred to intra-ovarian embryos. A total of 11 pregnant females was sensitized. Ten were given a single scale homograft and one was given three successive homografts. The young were born from 10 to 30 days after the parent had received the last graft. The newborn animals each received a scale homograft one to three days after birth

TABLE II

Host aged 1-3 days		Host aged 15 days		Host aged 1-3 days		Host aged 1-3 days	
Post-partum. Parent not sensitized.		Post-partum. Parent not sensitized.		Post-partum. Parent given 1 or 2 successive homografts.		Post-partum. Parent given 3 successive homografts.	
Specimen no.	Reaction time	Specimen no.	Reaction time	Specimen no.	Reaction time	Specimen no.	Reaction time
S-6-59-1a	16	S-6-59-7a	8	S-8-59-1a	7	S-8-59-19a	7
1b	16	7b	10	1b	7	19b	7
1c	16	7c	10	1c	7	19c	7
S-6-59-2	5	7d	10	1d	9	19d	7
3	15	7e	10	1e	9	19e	7
4	12	7f	10	S-8-59-2	13	19f	9
6a	12	7g	10	S-8-59-7	6	19g	9
6b	12			S-8-59-9a	3	19h	9
6c	12			9b	9	19i	11
6d	12			S-8-59-10a	9		
6e	17			10b	11		
				S-8-59-13a	8		
				13b	8		
				13c	10		
				S-8-59-14a	8		
				14b	11		
				14c	11		
				14d	11		
				14e	11		
				S-8-59-18a	8		
				18b	8		
				18c	10		
				18d	10		
				18e	10		
				S-8-59-21a	8		
				21b	8		
				21c	8		
				21d	11		
				S-8-59-22a	4		
				22b	6		
Average reaction time	13.2	9.7		8.6		8.1	
Total no. specimens	11	7		30		9	

from the same donor that had been used for the parent. The results are given in the third and fourth columns of Table II. The results for the progeny of singly grafted parents are tabulated separately from those for the progeny of the triply grafted parent. By comparing column one with columns three and four it can be seen that the reaction time for one- to three-day young from sensitized parents (average reaction time = 8.6 and 8.1 days) is significantly shorter than that for young of comparable age from unsensitized parents (average reaction time = 13.2

days). It can also be seen by comparing columns three and four that the parent with three successive grafts produced young with slightly greater sensitivity (average reaction time = 8.1 days) than did the parents with a single graft (average reaction time = 8.6 days). The evidence leaves little grounds for doubt that homograft sensitized parents can transfer this sensitivity to intra-ovarian young.

DISCUSSION

These experiments indicate a basic similarity between adult *C. aggregata* and *Carassius auratus* (Hildemann, 1957b) concerning the homograft response. Both species reject homografts, and both can be sensitized by successive grafting. In both species the time for homograft rejection is shortened by increase in temperature. Barrymore (unpublished data) has made similar observations on adult swordtails (*Xiphophorus helleri*) as have Triplett and Barrymore (unpublished data) on *Amphistichus argenteus* and *Hyperprosopon argenteum*. Fin transplantations on xiphophorin fishes yield similar results (Kallman and Gordon, 1957). There is good reason to suspect that these phenomena could be observed in teleosts in general.

To the authors' knowledge only one publication to date has dealt with tissue transplantation in older embryonic or immature fishes. Humm *et al.* (1957) have transplanted melanomas from adult platyfish-swordtail hybrids to embryos of both species and their hybrids. These workers have found, in contrast to the present authors, that homografts on embryonic fishes will not survive. This difference in results could be explained in one or both of two ways. First, it should be noted that the tissues grafted by Humm and his colleagues were not normal and should not be expected to behave as such. The inability of the transplanted melanomas to survive could be interpreted as a physiological deficiency other than immunological incompatibility.

A second and more likely possible explanation must take into account the different developmental rates of platyfishes and swordtails as compared with *C. aggregata*. The young of the former two species become free-living in about three weeks (Hooper, 1943), whereas the latter spends about five months in the ovarian cavity of the parent. A close correlation has been noted between the time of parturition in mammals (*e.g.*, Freund, 1930), hatching in birds (*e.g.*, Murphy, 1914; Waterman, 1936) or metamorphosis in amphibians (Schwind, 1933, 1937; Triplett, 1958) and the development of the ability to react immunologically to implanted or injected foreign materials. If our interpretation of Humm's work is correct those animals that were not killed by mechanical blockage, resulting from a rapidly proliferating graft, retained the transplant until well after the normal time of birth. The present experiments show that in *C. aggregata* homograft incompatibility is expressed soon after birth. This is quite possibly also the case in xiphophorin fishes used by Humm *et al.*

There has been considerable speculation as to whether or not circulating antibody is responsible for tissue incompatibility (reviewed by Snell, 1957). The first clear proof of an immune reaction to homografts was published by Gorer (1937) who found that tumor homografts could elicit the formation of circulating antibodies capable of agglutinating donor red cells. More recent work (see Snell, 1957) has established that humoral antibodies are formed in a variety of species

against a variety of transplanted tissues. Santos *et al.* (1959) have shown that the serum of graft-sensitized rodents, upon injection into other animals, can produce a passive heterograft sensitivity.

The present experiments on transfer of homograft sensitivity from parent to intra-ovarian embryos can be explained best by assuming that circulating antibody is produced against scale homografts. It is probable that antibody accumulates in the ovarian fluid and is ingested by the embryos (no placental connection exists). This hypothesized method of transfer is made more plausible by the fact that Hemmings and Morris (1959) have been able to observe the absorption of antibody from the gut in young mice. In addition, Brambel *et al.* (1959) have observed that homologous antitoxin is absorbed from the uterine cavity to the fetal circulation in the rabbit.

Hosoda *et al.* (1955) have reported experiments of interest in this context. They found that hens injected with horse erythrocytes produce antibodies and transfer these antibodies to offspring.

SUMMARY

1. Embryonic and adult *Cymatogaster aggregata* have been subjected to scale homotransplantations.
2. Homografts are rejected after a short period of time. The time required for graft rejection is temperature-dependent.
3. Increased sensitivity to homografts results from successive transplantations.
4. Intra-ovarian embryos used in these experiments were not capable of rejecting scale homografts.
5. Immature free-living fish rejected homografts, but more slowly than adults.
6. Pregnant females can transfer homograft sensitivity to intra-ovarian embryos. It is hypothesized that circulating antibody is passed to the embryo via the ovarian fluid and the embryonic hindgut.

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SOME ASPECTS OF OSMOREGULATION IN EMBRYONIC AND ADULT CYMATOGASTER AGGREGATA AND OTHER EMBIOTOCID FISHES

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The Embiotocidae is a family of marine (with one exception) teleosts which is somewhat unique in that all species possess a truly viviparous type of development. The young remain in the hollow ovary of the parent for a period of about five months, during which time they increase in size from 0.24 mm. (fertilized egg) to about 42 mm. (newborn) (Eigenmann, 1890, 1894). It was learned while devising a technique for culturing the embryos *in vitro* (Triplett, unpublished) that, unlike the adults, they are capable of tolerating only very limited variation in the osmotic concentration of the external medium.

The object of this study was to learn more precisely how well these animals can regulate to variations in the concentration of the external medium both as adults and during development. In addition, preliminary experiments were performed in an effort to find out why the adults are better able to regulate than the embryos.

MATERIALS AND METHODS

Experimental animals

Cymatogaster aggregata (Gibbons) was the most easily obtained of the embiotocids and was therefore the principal organism used in the investigation. This species is widely distributed over the west coast of North America, ranging from southern Alaska through Baja California (Tarp, 1952). It has been observed in estuaries where the salinity is quite variable (Hubbs, 1917) as well as in bays and along the open coast. Animals used in these experiments were collected by angling from Goleta Pier, Goleta, California.

Less extensively investigated were *Amphistichus argenteus* (Agassiz) and *Micrometrus minimus* (Gibbons). These species have about the same distribution as *C. aggregata*, but so far as the authors are aware, they do not enter brackish water. For these experiments they were collected with a beach seine on the campus beach at Goleta, California. Only adults of these two species were used.

Methods

The general approach was to determine the total osmotic pressure of the serum and the change in body weight after exposure to experimental salinities for periods ranging from six hours to nine days.

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The experimental animals were divided into four groups on the basis of their stages of development. Group 1 comprised intra-ovarian embryos ranging in standard length from 18 mm. to 25 mm. At this stage the animals were completely transparent except for a thin band of melanophores along the lateral line and a few scattered melanophores in the head region. The fins were hypertrophied and possessed highly vascularized dermal flaps between the rays. The hindgut was also hypertrophied and protruded in the cloacal area. The fins and hindgut probably serve as a pseudo-placenta (Eigenmann, 1890, 1894). The trailing edge of the caudal fin was convex or flat. Group 2 consisted of intra-ovarian embryos measuring between 27 mm. and 31 mm. in standard length. These animals still possessed hypertrophied fins and a hypertrophied protruding hindgut. Melanin deposition had increased considerably, and guanophores appeared on the dorsal half of the animal, rendering it semi-opaque. Scales had differentiated visibly at this time. The trailing edge of the caudal fin was becoming concave. Group 3 comprised intra-ovarian animals nearing parturition age and ranging in standard length from 32 mm. to 38 mm. The body was completely opaque and the adult pigment pattern was acquired. The fins, though not yet of adult proportion, had begun to atrophy. The hindgut had also atrophied and protruded only slightly. The trailing edge of the caudal fin was deeply concave and V-shaped as in the adult. Group 4 comprised newborn animals and adults measuring between 39 mm. and 115 mm. standard length. Preliminary experiments on group 4 animals of different lengths (ages) established that their osmotic responses were identical.

A minimum of four animals from each group was placed directly from holding tanks (running sea water) or directly from the ovarian cavity into each of the following solutions of sea water diluted with distilled water: 100% ($\Delta = 1.86$), 75%, 50%, 25%, 20%, 15%. For each series of measurements four adult animals were kept in an aerated battery jar containing 3000 ml. of fluid and maintained at 16° C., or four embryos were kept aseptically in a fingerbowl containing 1000 ml. of fluid and maintained at the same temperature. Serum freezing point determinations and weight measurements were made on four different animals of each group at each dilution after six hours and one, two, four and eight or nine days. Freezing points are here given in sodium chloride equivalents.

Weight determinations were made on blotted animals with a triple beam balance to an accuracy of 0.01 gram. All animals in group 4 were weighed before and after exposure to the experimental medium. Weight measurements were not possible with groups 1, 2, and 3 because these animals will not live after such handling.

Blood for freezing point determinations was taken by cardiac puncture or by removal of the tail at the level of the caudal peduncle. In the adults the particulate matter was removed before placing the serum in capillaries. This was not possible with the embryos since too little blood was available. Instead the freezing point capillary was inserted into the heart, and whole blood was drawn into the tube. The red cells and the clot were then packed by centrifuging, thus rendering the serum clear enough for freezing point determinations. Freezing points were determined with an apparatus modified after one used by Gross (1954), and since the modification offers some advantages over the original it is briefly described here. It consists of a Fiberglas-lined, insulated box containing four meters of one-half inch copper tubing. Two glass windows allow light to be passed through

the box. The box is filled with brine, and the brine is cooled by circulating freon-14 through the copper tubing. When the desired temperature is obtained the freon-14 is turned off, and the previously frozen capillaries containing the fluid to be measured are inserted in the brine between the observation windows. As the box warms very slowly the fluid in the capillaries melts. The melting point can be observed with great accuracy ($\pm 0.02^\circ \text{C.}$) by using crossed polaroids.

In an effort to learn something about differences in ionic exchange between different age groups preliminary experiments have been performed using chlorine-36 as a tracer. The technique consisted of placing the experimental animal, after peritoneal injection of radio-chloride, in a divided chamber so that the gills and half the body were on one side. Aliquots of the fluid in the two compartments were measured at intervals over a three- to eleven-hour period for radioactivity. The fluid in both compartments was a modified White's (1954) medium (.220 M/L NaCl equiv.) slightly hypertonic to the serum.

RESULTS AND DISCUSSION

Changes in serum freezing point and body weight in experimental media

Cymatogaster aggregata: The results of freezing point determinations and weight measurements on adult (group 4) *C. aggregata* are summarized in Figure 1, each point on the graph representing the average of measurements made of at least four fishes. It can be seen that the viable range of salinities for this species is quite large, ranging in these experiments from 20 per cent to 100 per cent sea water. Adults remained alive no longer than three hours in 15 per cent sea water, during which time they increased 3.83 per cent in weight.

At all salinities below 100 per cent sea water there was an initial drop in serum tonicity which reached a minimal value between one and two days after exposure to the experimental media. Accompanying this was a corresponding increase in wet weight. In general, the minimal serum osmotic value decreased as the salinity of the external medium was decreased. Less convincingly, Figure 1 indicates that as the salinity of the external medium is decreased, the time required for return to normal weight and serum tonicity increases. At all salinities tested above 20 per cent sea water, regulation is complete or nearly complete within nine days.

It will be noted that there was a small temporary increase in serum tonicity and decrease in wet weight of fishes maintained in 100 per cent sea water. The authors attribute this to an increased permeability of the integument resulting from handling. It is presumed that a similar increase in integumentary permeability was also experienced by fishes kept in other sea water dilutions, and this must be kept in mind when interpreting the results.

The results of freezing point determinations on the various embryonic stages are summarized in Table I, and corresponding figures for the adults are included for comparison. It can be seen that the viable range of salinities for embryos increases with increase in age. Group 1 and group 2 were able to survive for the complete duration of the experiment only in media between 25 and 36 per cent sea water (36 per cent sea water is equivalent osmotically to the ovarian fluid in which the animals normally reside), whereas group 3 was capable of regulation at all salinities between 25 and 75 per cent sea water.

However, it was noted that, in general, it required a greater alteration of the

serum osmotic potential of embryos than of adults to kill the animals. In these experiments group 1 and group 2 animals died when this value was above .232 M/L or below .114 M/L . Group 3 animals died at values above .223 M/L or below .156 M/L . Possibly for this reason the embryos live longer at 15 per cent sea water than do the adults. This may be a reflection of the fact that less differentiated systems are generally more tolerant of their physiological environment.

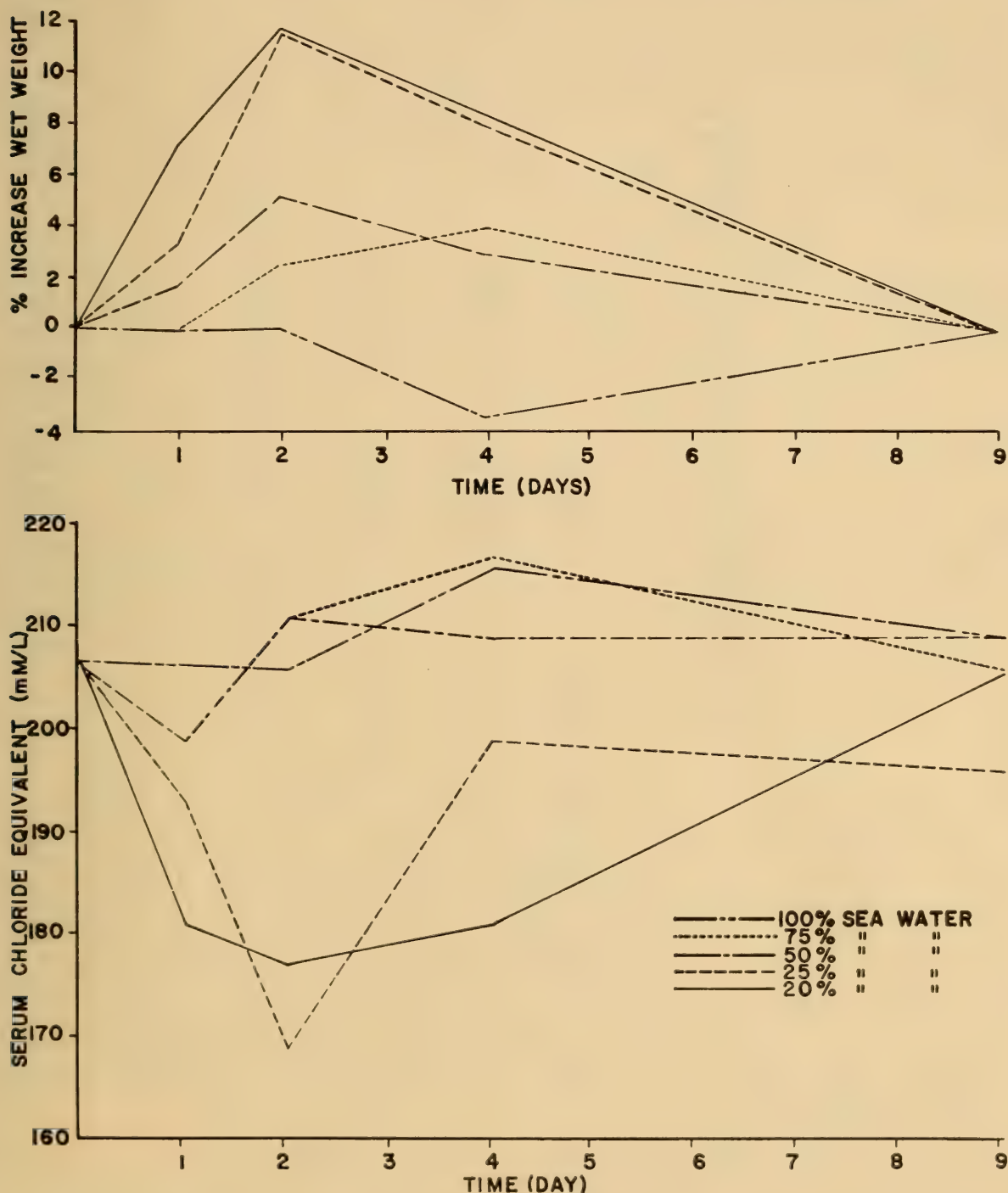


FIGURE 1. Upper graph: the wet weight of *C. aggregata* adults plotted as a function of time media of different salinities. Lower graph: tonicity (sodium chloride equivalents) of *C. aggregata* adult serum plotted as a function of time in media of different salinities.

TABLE I

Serum tonicity of C. aggregata of different developmental stages after exposure to media of variable salt concentrations for variable intervals of time

External medium							
% Sea water NaCl equiv. (M/L)		100 .568	75 .326	50 .284	25 .142	20 .114	15 .085
Age group	Time exposed						
1. Transparent	6 hours	death	death	.230	.160	.135	.120
	1 day			death	.164	.131	.115
	2 days				.185	death	death
	4 days				.192		
	6 days				.190		
	8 days				.195		
2. Semi-opaque	6 hours	.232	.210	.205	.160	.135	.125
	1 day	death	death	.245	.156	.138	.114
	2 days			death	.181	.158	.125
	4 days				.185	death	death
	6 days				.190		
	8 days				.195		
3. Opaque	6 hours	.227	.198	.196	.192	.198	.179
	1 day	.233	.202	.209	.191	.197	.179
	2 days	death	.236	.221	?	?	death
	4 days		.221	.215	.195	.156	
	6 days		?	?	?	death	
	8 days		.211	.209	.206		
4. Adult	6 hours	?	?	?	?	?	death
	1 day	.206	.199	.193	.193	.181	
	2 days	.206	.211	.211	.169	.177	
	4 days	.216	.217	.209	.199	.181	
	6 days	?	?	?	?	?	
	9 days	.209	.206	.216	.196	.209	

Micrometrus minimus and *Amphistichus argenteus*: Similar though less extensive experiments were performed with the adults of these two species. It was expected that since they do not enter brackish water, the ability of *M. minimus* and *A. argenteus* to regulate to differentiate salinities would be less than that of *C. aggregata*, but this was not the case. Both were quite capable of remaining alive in media between 20 and 100 per cent sea water over the nine-day interval. Furthermore, serum freezing points of animals living in any of these dilutions at the end of this time were the same as those of animals living in the normal environment (serum = .209 M/L).

Ion exchange studies using Cl³⁶ tracers

The decreased ability of embryos to regulate to different tonicities of the external medium as compared to adults could be explained in several ways. One possible explanation is that these animals may not yet have differentiated the

branchial salt secretory mechanism that has been demonstrated in various adult teleost fishes (Smith, 1930). Both adults and embryos of *C. aggregata* were examined for this mechanism by utilizing the divided box technique first used by Smith (1930). The animals were injected intraperitoneally with 0.25 ml. of an isotonic salt solution with an activity of 0.125 microcuries per milliliter. At two-hour intervals or less, 250-lambda samples were withdrawn from the anterior and from the posterior chambers and measured for radioactivity with an accuracy of 1.0 per cent.

Three adults ranging in standard length from 105 mm. to 115 mm. and three sibling embryos (group 3) measuring 33 mm. were used. The results for one animal of each group are shown in Figure 2. The general results were about the same for any animal in a given age group. It can be seen that in the adults the activity of the anterior chamber is consistently higher than that of the posterior for the duration of the experiment. This would indicate that a branchial salt secretory mechanism is operative. However, in the embryos, the activity of the posterior was consistently higher than that of the anterior chamber. This is tentatively interpreted to mean that the "salt pump" in the gills is not yet in operation or is operating at a much reduced rate. Though the embryos were placed in the chamber so that half the body was on each side, it was roughly estimated that 74 per cent of the body surface area was contained by the posterior chamber. The hypertrophied fins account for the greater part of this area. This could account for the higher Cl^{36} value in the rear chamber by assuming that chloride loss is about equal over the whole body surface (including the gills).

These tracer studies are quite obviously not sufficient to draw definite conclusions. The sample is small, and desirable control experiments have not been performed. The experiments do suggest, however, that the "salt pump" operative in the adult is absent or operating less efficiently in the embryo. Further efforts in this direction are planned.

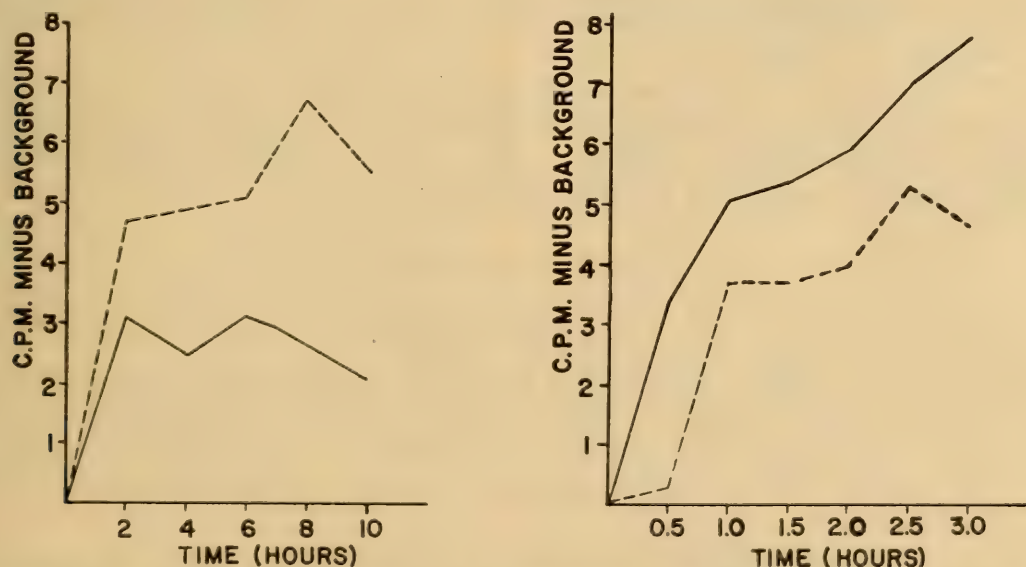


FIGURE 2. Radioactivity in front and back chambers of a divided box so arranged that the anterior half of a chloride³⁶-injected fish is contained in the front chamber and the posterior half of the fish is contained in the back chamber. Dotted lines = front chamber; solid lines = back chamber. Left graph = measurement for adult fish; right graph = measurements for embryonic fish.

Another clue concerning the lack of osmoregulatory ability in embryos was gained by observing the volume of the hindgut lumen in media of different osmotic concentrations. It was noted that embryos placed in any of the hypertonic solutions ($> 36\%$ sea water) experience a swelling of the hindgut. The hindguts of animals placed in hypotonic solutions shrink. It is well known that marine teleosts maintain osmotic equilibrium by drinking sea water and excreting the excess salt, principally through the gills. It is hypothesized that these embryos also drink the external medium. If they should be placed in a hypertonic medium there would be created an osmotic gradient between body fluids and hindgut lumen resulting in body dehydration and swelling of the hindgut lumen. The opposite gradient would result if a hypotonic medium were drunk by the embryo. The question remains as to why this does not happen in the adult.

SUMMARY

1. The osmoregulatory abilities of adult and embryonic *Cymatogaster aggregata* were tested by determining serum freezing points and weight changes after exposure to dilutions of sea water ranging between 15 and 100 per cent.

2. Adults were able to regulate in external media ranging between 20 and 100 per cent sea water. The greatest deviations of weight and serum freezing point from the normal occur after one to two days in the experimental media. Regulation in all media is complete at or before nine days of exposure.

3. The ability of embryos to regulate is proportional to their stage of development. The youngest animals tested regulate only in media with values between 25 and 36 per cent sea water.

4. Similar though less extensive experiments were performed with adult *Amphistichus argenteus* and *Micrometrus minimus*. The osmoregulatory ability of these species is identical to that of adult *C. aggregata*.

5. Preliminary experiments using Cl^{36} as a tracer indicate that the branchial salt secretory mechanism present in the adults is absent or operating less efficiently in the embryos of *C. aggregata*.

6. Observations on the volume of the gut in different external media indicate that the embryos drink the medium, thus creating an osmotic gradient between the body fluids and the lumen of the gut. The result is dehydration or "edema," depending upon the tonicity of the external medium.

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